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TITLE

Immunotherapy in hiv infected persons using vaccines after multi-drug treatment

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either filled or decorated with a desired immunogen of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the immunogen(s).

Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, *e.g.*, liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, *e.g.*, Szoka, *et al.*, *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which could be changed or modified to yield essentially similar results.

Example 1. Administration of NYVAC-SIV$_{gag\text{-}pol\text{-}env}$ to SIV-infected, HAART-treated *Rhesus macaques*

The effectiveness of a highly attenuated Poxvirus vector as a therapeutic vaccine to enhance host-immune responses was investigated in the SIV$_{251}$ Rhesus macaque model, which models HIV-1 infection in humans. The vaccine used for this study was a highly attenuated NYVAC-SIV *gag-pol-env* recombinant vaccine that was demonstrated to have efficacy as a preventative vaccine in earlier studies (Benson *et al.*, *J. Virol.* 72:4170-4182 (1998).

The study design included 24 animals which were divided into three groups, A, B, and C. All the animals were infected intraveneously with ten infectious doses of highly pathogenic SIV$_{251}$ (Pal). Following SIV$_{251}$ exposure, all twenty four

animals became infected; the peak of plasma viremia occurred at approximately two weeks and ranged between 10^7 and 10^9 copies of viral RNA/ml of plasma in the twenty four animals.

Two and a half weeks after infection, sixteen animals, those in groups A and B, received a HAART regimen that in a pilot study had reduced viremia to undetectable levels in 80% of macaques chronically infected with SIV_{251}. The HAART regimen included oral administration of two doses of Stavudine (1.2 mg/day), intravenous inoculation of DDI (10 mg/kg/day) and subcutaneous inoculation of PMPA (20 mg/kg/day). Animals in group C were not treated with drugs. Animals in groups A and B, but not C, experienced a significant decrease in viremia (Figure 1, top panels).

In the sixteen animals of groups A and B, HAART treatment was continued daily for 6 months. At weeks 10, 19, and 23 post-infection, the animals in group A received a placebo vaccine (non-recombinant NYVAC vector) and the animals in groups B and C received 10^8 pfu of NYVAC-SIV *gag-pol-env* vaccine. All twenty-four animals were monitored weekly for viral RNA copies/ml of plasma and biweekly for lymphoproliferativ responses (LPR) to highly purified native p27 *gag* and gp120 *env* SIV proteins.

In order to follow the $CD8^+$ T-cell responses, three MAMU-A*01 (*Macaca mulata* equivalent of HLA class I A*01 (Kuroda *et al., J. Exp. Med.* 187:1373-1381, 1998) were included in each group. MAMU-A*01 animals are generally able to recognizes the immunodominant peptide 11c-m within the gag antigen of SIV. A tetramer binding assay was therefore used to directly quantitate $CD8^+$ T-cell responses *in vivo*. The tetramer, formed by four identical MAMU-A*01 molecules conjugated to the peptide 11c-m, was linked to fluorescent-labeled streptavidin and used to stain the $CD8^+$ T-cells in the blood of macaques that expressed the appropriate T cell receptor complex on their surface. The percentage of total $CD8^+/CD3^+$ staining with the peptide MAMU-A*-1 tetramer was measured in several consecutive time intervals in each of the MAMU-A*01 animals.

Staining of fresh or cultured PBMC with the MAMU A*01/peptide 11c-m tetramer was performed in all the nine MAMU A*01 animals included in the study (three in each group) following SIV_{251} infection and after the second NYVAC-SIV vaccination. MAMU-A*01/peptide-11c-m-tetramer-staining $CD8^+$ T-cells (ranging from 0.8 to 4.6%) were induced by SIV infection in all nine animal within the first

month after viral exposure, and the $CD8^+$ T-cell population was expanded (up to values of approximately 70%) *in vitro* following specific peptide 11c-m stimulation. At two months after SIV_{251} exposure, the infected macaques seroconverted to SIV_{251} antigens, including the animals in groups A and B that were treated with HAART. Measurements of LPR to gp120 and p27 *gag* was consistently negative in all twenty four animals within the first four weeks following viral infection.

The frequency and extent of $CD4^+$ T-helper responses in HAART-treated animals is increased by NYVAC-SIV vaccination.

As stated above, acute infection by SIV_{251} was associated with the absence of a proliferative response to both p27 *gag* and gp120 *env*. However, responses to p27 *gag* appeared in HAART-treated animals at approximately 10 weeks after infection and, these responses were more frequent in animals of group A than in animals of group C. No difference in the LPR to gp120 was observed between these two groups (Figure 1, middle and lower panels).

This notion was further supported by the finding that two animals (647 and 655) in group B failed to respond to therapy, maintained high virus load, and did not develop CD4+ T-cell proliferative response following NYVAC-SIV vaccination.

Further corroborating this notion, two animals in group C that naturally controlled viremia developed LPR following NYVAC-SIV vaccination. Thus, it appears that $CD4^+$ T-helper memory responses are induced inefficiently by NYVAC-SIV animals with high viremia. Several factors may contribute to this finding: $CD4^+$ T-cells are already activated *in vivo* and do not further proliferate *in vitro* in LPR assays and/or the vaccine-induced memory cells become targets for SIV infection and die upon further antigen stimulation. These data provide the first evidence that a highly attenuated live recombinant poxvirus vector vaccine can induce and boost sustained $CD4^+$ helper immune response in the context of a pharmacologically controlled lentiviral infection.

*NYVAC-SIV vaccination increases $CD8^+/CD3^+$ MAMU-A*01-tetramer-positive cells only in HAART-treated animals.*

SIV infection induced a large number of $CD8^+$ T-cells that bound the MAMU-A*01 tetramer. This response by week four was reduced in most animals. Following the second and third NYVAC-SIV vaccinations, a high percentage of $CD8^+/CD3^+$ T-

cells bound tetramers in the fresh PBMC of all MAMU-A*01 animals in group B, but in none of the animals in groups C (Figure 2). The specificity of tetramer staining was shown in parallel experiments using PBMC from animals with a different haplotype as well as by the expansion of the cells from the nine MAMU-A*01 animals *in vitro* following peptide-11c-m-specific stimulation. Peptide 11c-m presented in the context of the MAMU-A*01 haplotype is recognized by $CD8^+$ T-cells with cytolytic activity. (In Figure 2, the top panel shows the results obtained in the three MAMU-A*01 animals from group A; middle panel, from group B; and bottom panel, from group C. The percentage of MAMU-A*01/peptide-11c-m-tetramer-staining cells within the first 4 weeks was evaluated using only α-$CD8^+$ antibodies as a T cell marker whereas the data presented from weeks 19 through 29 were obtained using simultaneously α-CD-3+ and α-$CD8^+$ antibodies in conjunction with the MAMU-A*01/peptide-11c-m tetramers. For some of the MAMU-A*01-positive animals, CTL activity obtained in cultured PBMC [weeks 19 and 20] or fresh PBMC [week 23] is also presented. The numbers in the abscissa represent the effector-target ratio of the CTL assay system.)

To assess whether the detection of MAMU-A*01/peptide-11c-m-tetramer-staining $CD8^+/CD3^+$ T-cells in the PBL of macaques was associated with CTL activity, cytotoxicity assays were performed using homologous B cells from each animal pulsed with peptide 11 c-m. It was shown that CTL activity was measured after *in vitro* stimulation of $CD8^+$ T-cells in all animals tested at weeks 19 and 20, although the extent of killing did not correlate with the percentage of tetramer-staining cells (Figure 2). Most notably, CTL assays performed on fresh $CD8^+$ T cells at day 23 demonstrated significant CTL activity in group B animals, confirming the CTL functional activity of the high number of circulating $CD8^+$ tetramer-staining cells in the blood of these animals (Figure 2). Thus, NYVAC-SIV vaccination induced high levels of $CD8^+$ responses only in animals in which viral replication was suppressed by therapy.

Delayed T-cell hypersensitivity (DTH) to viral p27 gag

Vaccines able to induce T-cell-mediated immunity are often able to induce DTH. To assess whether any of the animals vaccinated with NYVAC-SIV developed this response, either 1 or 10 ug of highly purified SIV p27 or HTLV-I p24, as controls, were inoculated

intradermally in animals in groups B and C. DTH reactivity was considered positive when a thickness of more than 10 mm manifested at 72 h postinoculation. Only three animals in group B and two animals in group C fulfilled the requirement for DTH positivity.

The data presented in this example demonstrated that inoculations of NYVAC-SIV following HAART greatly increased the frequency, extent, and duration of those responses in animals in which viremia was efficiently suppressed, indicating that the ability to detect vaccine-induced $CD4^+$ T-cell helper responses was strictly dependent on the level of viral replication in the host. Similarly, NYVAC-SIV vaccination induced significant expansion of the number of $CD8^+/CD3^+$ MAMU-A*01 cells specific for an immunodominant SIV *gag* peptide only in animals treated effectively with antiviral therapy. Following therapy suspension, NYVAC-SIV-vaccinated animals were able to control viremia better than animals treated with antiviral therapy alone. These data demonstrate that vaccination can further induce both $CD4^+$ and $CD8^+$ T-cell responses in SIV-infected macaques.

Example 2. Immunization of a person infected with HIV.

A 35 year old male patient is seropositive for HIV and has a viral count in his plasma of 15000 copies per milliliter and a $CD4^+$ count of 300. The patient is treated with a cocktail of antiviral agents which consist of: two nucleotide inhibitors and one protease inhibitor (Zaduvidine [three time a day], Lamivudine [two times a day], and Nesinavir [three times day] at preformulated doses. Typically, antiretroviral therapy is considered effective if a decrease of at least one log of the plasma viral RNA is observed. The plasma RNA load as well as the $CD4^+$ T-cell count in the blood will be measured monthly.

After six months the patient is re-evaluated and is determined to have a viral count in his plasma of 1500 copies per milliliter or less and a $CD4^+$ count of 500. He is then injected with an attenuated pox virus vector NYVAC carrying the following viral peptides: *gag*, *pro*, gp120-TM, *pol* and *nef* string of CTL epitopes. The injection comprises 10^8 pfu of the pox virus.

The patient's immune responses is evaluated (CD4+ proliferative response; cytotoxic CD8+ T-cell activity, etc.) and a decision is made as to whether and when to immunize again. Typically, a maximum of three to four immunizations with NYVAC-HIV-1 is considered. This regimen could be followed by three to four immunizations with ALVAC-HIV-1 (carrying a similar HIV-I genetic content) and, if

necessary, an additional regimen of DNA-only *i.e.*, DNA that is not in a viral vector, immunization could follow. NYVAC-HIV-1 followed by ALVAC-HIV-1 is effective in inducing immunoresponses in chimpanzees. Similarly, immunization regimens as follows have been shown to be effective: NYVAC followed by DNA and ALVAC followed by DNA. Thus, a vaccination regimen including all of the above vaccines may be used.

Often, the vaccine regimen is administered with IL-2, preferably at low doses such as 100,000 to 200,000 units of IL-2 administered daily. $CD40^+$ ligand can also be included in the treatment protocol, either by itself or administerd in conjunction with the IL-2 treatment.

Example 3. Administration of ALVAC-SIV_{gpe} to SIV-infected, HAART-treated *Rhesus macaques*

The effectiveness of a highly attenuated ALVAC vector as a therapeutic vaccine to enhance host-immune responses in HAART-treated animals was investigated in the SIV_{251} *Rhesus macaque* model, which models HIV-1 infection in humans. The study includes 16 macaques inoculated with the SIV_{251} virus and treated with the HAART regimen as described in Example 1 at day 15 and thereafter. Of those macaques, 8 are immunized with a total of 3 doses of 10^8 pfu of the mock-vaccine ALVAC vector (group D) and the remaining 8 (group E) with a recombinant ALVAC-SIV-*gag-pol-env* vector (ALVAC-SIV_{gpe}), which is analogous to the NYVAC vector of Example 1.

The data obtained after a single dose of the ALVAC-SIV_{gpe} vaccine indicated that the ALVAC-SIV_{gpe} vaccine is able to boost $CD4^+$ T-cell responses against the p27 Gag protein as well as to the gp120 Env forefront of the vaccine (see Figure 3). In addition, a specific $CD8^+$ T-cell response detected using the MAMU A*01/peptide 11c-m tetramer reagent (*see, e.g.*, Example 1) was also boosted by the ALVAC-SIV_{gpe} (Figure 4), and further, those $CD8^+$ T-cells could be expanded in culture. The mock-vaccinated animals did not experience an expansion of these immune responses.

Thus, ALVAC-SIV_{gpe} is immunogenic in animals undergoing HAART therapy. An ALVAC vaccine analogous to the NYVAC vaccine of Example 2 can similarly be employed to treat HIV-infected individuals as described in Example 2 who are undergoing HAART.

Example 4. MVA-SIV-*gag-pol-env* immunization of SIV-infected macaques.

The effectiveness of an MVA-SIV vaccine was evaluated using methodology analogous to that used to evaluate NYVAC and ALVAC SIV *gag-pol-env* vaccines. The CD8+ and $CD4^+$ responses were determined in SIV_{251}-infected macaques that were able to control viremia, *i.e.*, the CD4+ T-cell counts were above 500, following administrations of a single dose of 10^8 pfu of MVA-SIV-*gag-pol-env* recombinant vaccine. The results showed that both $CD4^+$ and $CD8^+$ responses could be expanded in infected animals.

Thus, MVA-SIV-*gag-pol-env* is also immunogenic in animals and can be used in patients undergoing HAART therapy.

Example 5. Comparison of the Immunogenicity of DNA and NYVAC vaccines.

A vaccine regimen comprising administration of DNA alone or DNA in combination with NYVAC-SIV or ALVAC-SIV, or administration of a combination of NYVAC-SIV and ALVAC-SIV is also effective in continuous boost of the immune response in SIV_{251}-infected animals.

A study conducted in parallel compared 2 inoculations of 10^8 pfu of NYVAC-SIV_{gpe} and 3 inoculations of DNA, which was administered using 4mg intramuscularly and 1 mg intradermally of each plasmid, in naïve animals. The DNA vaccine induced $CD4^+$ and $CD8^+$ T-cell responses that were equivalent to those induced by a NYVAC-SIV_{gpe} vaccine (Figures 5 and 6). ALVAC-SIV_{gpe} was at least as immunogenic as NYVAC-SIV_{gpe} (*see, e.g.*, Example 1) and NYVAC-SIV_{gpe} was as immunogenic as DNA. Thus, all three vaccines either alone or in various combinations can be used in HIV-I-infected individuals.

ALVAC-SIV_{gpe} was also able to induce both $CD4^+$ and $CD8^+$ T-cell response in chronically infected animals ($CD4^+$ T-cell range 50-900) and was able to suppress viremia in the absence of HAART. These animals, which had been previously vaccinated with a NYVAC-SIV_{gpe}-based vaccine, appear to model long-term progressor HIV-I-infected individuals. The vaccines of the invention can therefore be used alone or in various interchangeable combinations in early infection as well as late infection in individuals in which viremia is controlled pharmacologically or otherwise.

Example 6. Therapeutic Vaccination with NYVAC and IL-2.

This examples demonstrates the ability of immunomodulatory molecules, *e.g.* IL-2, to further expand both $CD4^+$ and $CD8^+$ T-cell responses induced

by the NYVAC-SIV$_{gag\text{-}pol\text{-}env}$ vaccine and increase the breadth of the host response to the virus.

SIV$_{251}$-infected HAART-treated Rhesus macaques (Figure 7) were vaccinated intramuscularly with NYVAC-SIV$_{gag\text{-}pol\text{-}env}$ with or without simultaneous and continued daily treatment with IL-2 (120,000 IU) administered subcutaneously. A control group of animals was treated with IL-2 and mock-vaccinated (NYVAC nonrecombinant vector). All 15 macaques in the study responded to HAART (10 mg/kg/day DDI, intravenously; 2.4 mg/kg/day Stavudine, orally; 10 mg/kg/day PMPA, subcutaneously) and in 13 animals viremia was suppressed below 5 x 10^3 copies/ml within the first 4 weeks of treatment. Viremia in the remaining 2 animals became undetectable by weeks 6 and 8 of treatment. Proliferative responses to p27 Gag and gp120 were increased by NYVAC-SIV$_{gag\text{-}pol\text{-}env}$ vaccination up to three- and twelve-fold, respectively, regardless of IL-2 treatment, indicating that either IL-2 does not increase bulk proliferative response or that the assay was not sensitive enough to measure subtle variation in the antigen-specific CD4$^+$ T-helper response.

Approximately half of the animals in the study were genetically selected as carriers of the Mamu-A*01 molecules. CTL CD8$^+$ T-cell responses to SIV$_{251}$ were therefore measured by ELISPOT using several purified SIVmac$_{239}$ nonamer peptides and their corresponding Mamu-A*01 tetramers. The *ex vivo* PBMC of all SIV$_{251}$-infected Mamu-A*01 animals recognized the immunodominant p11C,C→M peptide and produced γ-interferon (γ-INF) following *in vitro* stimulation. This response was further expanded following immunization with NYVAC-SIV$_{gag\text{-}pol\text{-}env}$. While IL-2 did not expand the number of γ-INF-producing cells in response to the p11c, C→M peptide in mock-vaccinated animals, the expansion of this response was higher in the NYVAC-SIV$_{gag\text{-}pol\text{-}env}$-treated macaques that also received IL-2 than in those that received NYVAC-SIV$_{gag\text{-}pol\text{-}env}$ alone. In contrast, IL-2 *per se* appeared to expand the immune response to two other immunodominant epitopes within the SIV tat and vif proteins in treated animals. (Vaccination with NYVAC-SIV$_{gag\text{-}pol\text{-}env}$ did not further expand these responses as these antigens are not included in the vaccine.) Moreover, the CD8$^+$ T-cell responses to 2 subdominant epitopes within the Gag and Env proteins of SIV were clearly expanded following NYVAC-SIV$_{gag\text{-}pol\text{-}env}$ vaccination in macaques that received simultaneous and continuous IL-2 treatment. Thus, the administration of low-dose IL-2 in conjunction with vaccination with the

highly attenuated NYVAC-SIV$_{gag\text{-}pol\text{-}env}$ vaccine potentiated and broadened CD8$^+$ T-cell functional responses to SIV$_{251}$.

IL-2 can be used with the vaccine to control viremia after antiretroviral therapy interruption

A HAART-treated macaque was inoculated with NYVAC-SIV*gag-pol-env* at the intervals shown in Figure 8. The macaque also recived low dose IL-2, *i.e.*, 120,000 units daily administered subcutaneously. This animal exhibited expanded CD8$^+$ (top panel, Figure 8) and CD4+ proliferative (lower panel, Figure 8) responses. Furthermore, as shown in the top panel of Figure 8, transient viral rebound occurred following interruption of HAART treatment and after suspension of the IL2 treatment. Thus, administration of IL-2 in conjunction with vaccination can contribute to the control of viremia after interruption of antiretroviral therapy.

Improved Vaccine Protection from Simian AIDS by the Addition of Nonstructural Simian Immunodeficiency Virus Genes[1]

Zdeněk Hel,*† Wen-Po Tsai,* Elzbieta Tryniszewska,*‡ Janos Nacsa,* Phillip D. Markham,§ Mark G. Lewis,¶ George N. Pavlakis,‖ Barbara K. Felber,# Jim Tartaglia,** and Genoveffa Franchini[2]*

An HIV-1 vaccine able to induce broad CD4$^+$ and CD8$^+$ T cell responses may provide long-term control of viral replication. In this study we directly assess the relative benefit of immunization with vaccines expressing three structural Ags (Gag, Pol, and Env), three early regulatory proteins (Rev, Tat, and Nef), or a complex vaccine expressing all six Ags. The simultaneous administration of all six Ags during vaccination resulted in Ag competition manifested by a relative reduction of CD8$^+$ T cell and lymphoproliferative responses to individual Ags. Despite the Ag competition, vaccination with all six Ags resulted in a delay in the onset and a decrease in the extent of acute viremia after mucosal challenge exposure to highly pathogenic SIV$_{mac251}$. Reduced levels of acute viremia correlated with lower post-set point viremia and long-term control of infection. In immunized animals, virus-specific CD4$^+$ T cell and lymphoproliferative responses were preserved during acute viremia, and the maintenance of these responses predicted the long-term virological outcome. Taken together, these results suggest that the breadth of the immune response is probably more important than high frequency responses to a limited number of epitopes. These data provide the first clear evidence of the importance of nonstructural HIV Ags as components of an HIV-1 vaccine. *The Journal of Immunology*, 2006, 176: 85–96.

Human immunodeficiency virus type 1/SIV infection is associated with acute loss of CD4$^+$ T cells (1–3), a progressive demise of the immune system, and AIDS (4, 5). Although the host mounts strong cellular and humoral immune responses to HIV/SIV, the impairment of CD4$^+$ T cell help, the functional exhaustion of CD8$^+$ T cells, and the plasticity of the viral genome contribute to the inefficient control of viral replication and consequent disease progression. Thus, effective T cell vaccines for HIV/SIV should elicit CD4$^+$ and CD8$^+$ T cell responses of sufficient breadth and size to minimize viral replication and immune escape. Promising approaches to vaccine design have included combinations of heterologous vaccine modalities, because they appear to provide superior protection against HIV/SIV infection. Among these, the DNA/poxvirus combination has been demonstrated to be immunogenic in macaques and confer a degree of protection that depends on the dose and type of DNA, the level of expression of HIV/SIV Ags by the poxvirus vector, and the challenge virus used (6–11). In most of those studies, structural HIV/SIV Ags have been used, and the contribution of nonstructural Ags to protection remained unclear because head-to-head comparisons in a relevant animal model have not been performed.

The HIV/SIV genome encodes the structural/enzymatic proteins Gag, Pol (reverse transcriptase, protease, and integrase), and Env and the regulatory proteins Rev, Tat, Nef, Vif, Vpx, and Vpr. HIV-1 encodes an additional protein, Vpu, but lacks the open reading frame for Vpx. After viral entry, reverse transcription, and integration of the viral genome, the first genes expressed by HIV/SIV are completely spliced mRNAs that encode for the nonstructural Tat, Rev, and Nef proteins (12–14). Only when Rev reaches a sufficient level are the incompletely spliced Env mRNA and the genomic RNA encoding for Env and Gag-Pol, respectively, transported to the cytoplasm for translation. Being expressed early in the viral life cycle, Tat, Rev, and Nef proteins may be recognized by the immune system early in infection. Indeed, cytotoxic responses to epitopes encoded by these open reading frames are frequently detected in primary HIV infection (15, 16). Immune response to early regulatory genes may be particularly important to viral containment, because their recognition by cytotoxic CD8$^+$ T cells may occur before Nef-mediated down-modulation of MHC class I molecules on the surface of infected cells, resulting in the elimination of infected cells before the release of virions (17–22). The ability of early regulatory protein-specific CTLs to exert immunological pressure has been inferred by the selection for viral immune escape variants in Tat (23) and Nef (24, 25) during primary SIV infection. In both macaques and humans, the presence of humoral and cellular immune responses against Tat (26–30) and of CTL responses against Rev (31, 32) and Nef (33) has been demonstrated to inversely correlate with disease progression.

The inclusion of regulatory proteins as part of vaccines against immunodeficiency viruses has been tested in both macaques and humans with variable results (34–43). Immunization with Tat alone or a Tat toxoid, for example, has conferred limited or no

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protection depending on the animal model and challenge virus (34, 35, 41, 44).

The use of unaltered *tat*, *rev*, and *nef* gene products as components of a vaccine candidate has possible limitations due to the intrinsic ability of early regulatory proteins to interfere with host immune response mechanisms by various mechanisms, including down-modulation of MHC class I and II, CD4, and CD28 proteins; induction of T cell unresponsiveness and apoptosis; and up-regulation of Fas ligand (17, 45–52). Furthermore, the small size of these proteins represents a limitation, because fewer epitopes have a chance to be recognized by a given MHC class I. In addition, the expression of HIV and SIV genes in mammalian cells has proven difficult because of a highly distinct codon bias for adenine and thymidine at the third codon position (53, 54), limiting their translational efficiency. To overcome the potential shortcomings of early gene-based vaccines, we designed a novel chimeric gene comprising genetically modified, attenuated, and reassorted *rev*, *tat*, and *nef* genes of SIV (*retanef*) that has been optimized for expression in mammalian cells (55).

To assess directly the contribution of nonstructural proteins to vaccine protection, we have performed a head-to-head comparison of the protective effect of DNA- and NYVAC-based vaccines expressing the chimeric Retanef protein alone (55), a combination of Gag, Pol, and Env proteins (56), or a combination of all six Ags together in the SIV_{mac251} macaque model. We used a DNA prime/poxvirus boost regimen with highly attenuated NYVAC-based recombinant SIV vaccines, because we have previously demonstrated that the regimen confers higher frequency of virus-specific $CD4^+$ and $CD8^+$ memory T cell responses than recombinant NYVAC-SIV alone (8, 9). In this study we demonstrate for the first time that the addition of modified early SIV regulatory Ags to structural SIV Ags results in a delay of primary viremia, reduces its extent, preserves virus-specific $CD4^+$ T cells, and increases survival of the immunized animals. In addition, the data presented in this study suggest that the simultaneous administration of multiple Ags in primates may result in Ag competition and decreased immune responses to individual Ags.

Materials and Methods

Animals, immunizations, and challenge

All animals were colony-bred rhesus macaques (*Macaca mulatta*) obtained from Covance Research Products. The animals were housed and handled in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International, and the study was reviewed and approved by the animal care and use committees at Advanced BioScience Laboratories and Bioqual. All rhesus macaques were seronegative for SIV-1, simian T cell leukemia/lymphoma virus type 1, and herpesvirus B before the study. Molecular typing of MHC class I alleles that bind SIV-derived peptides, namely, Mamu-A*01, Mamu-A*02, Mamu-A*08, Mamu-A*11, Mamu-B*01, Mamu-B*03, Mamu-B*04, Mamu-B*17, and Mamu-B*29, was performed using the technique of sequence-specific primer DNA amplification as previously described (57, 58). The 3′-terminal region of sequence-specific primer DNA amplifications targeted nucleotide polymorphism unique to rhesus MHC class I alleles. Large scale, low endotoxin plasmid DNA was prepared by Qiagen under good manufacturing practices specifications. All NYVAC virus-derived preparations were provided by Sanofi-Pasteur.

Group 1A animals were not immunized. The animals in control group 1B were immunized i.m. with 10^8 PFU of mock NYVAC at weeks 0, 4, 24, and 52. At weeks 0, 4, and 12, animals in groups 2, 3, and 4 were immunized with plasmid DNA in PBS (1 mg/ml) administered by i.m. and intradermal routes. SIV-Retanef (55) and Rev-independent SIV Gag and SIV Env (8) expression vectors optimized for high level expression in primate cells were described previously. Group 2 received 1 mg of Retanef-encoding plasmid (five doses of 0.2 ml each) intradermally into five different sites in the abdominal area and 1 mg of Retanef (four doses of 0.25 ml each) i.m. into two sites on each leg. For the third immunization, the dose of Retanef administered i.m. was increased to 3 mg (four doses of 0.75 ml each). Group 4 received a mix of 1 mg of Env and 1 mg of Gag plasmids (10 doses of 0.2 ml each) intradermally into 10 different sites in the abdominal area and 3 mg of Env and 3 mg of Gag (four doses of 1.5 ml each) i.m. into two sites on each leg. Group 3 was immunized with both Retanef and Env/Gag plasmids, as described for groups 2 and 4. At weeks 24 and 52, groups 2, 3, and 4 were boosted with 10^8 PFU of the appropriate NYVAC recombinant vaccines given i.m.; group 4 received NYVAC-SIV-*gag-pol-env* (NYVAC-SIV-*gpe*),[3] group 2 received NYVAC-SIV-*retanef* (NYVAC-SIV-*rtn*), and group 3 received both vaccines at the same dose (see Fig. 1).

The challenge virus stock was prepared from PHA-activated PBMCs obtained from Mamu-A*01-positive macaque 561L previously inoculated vaginally with SIV_{mac251} (59). The monkeys were challenged by intrarectal exposure to 30 mucosal infectious doses of SIV_{mac251} challenge stock. SIV_{mac251} RNA in plasma was quantified by nucleic acid sequence-based amplification (60). Data describing the immunization and challenge of animals in groups 1B and 4 were published previously (8, 9). Because the same batches of DNA, recombinant NYVAC viruses, and challenge virus as well as identical immunological and virological assays were used in all studies, data from previous experiments were used for comparison purposes in the current study.

Lymphocyte proliferation assay

The Ficoll-purified PBMCs were resuspended in RPMI 1640 medium (Invitrogen Life Technologies) containing 5% inactivated human A/B serum and antibiotics (Sigma-Aldrich), and cultured at 10^5 cells/well in triplicate for 3 days in the absence or the presence of native HPLC-purified SIV_{mac} Gag p27 or Env gp120 proteins (Advanced BioScience Laboratories) or Con A as a positive control. The cells were then pulsed overnight with 1 μCi of [^{3}H]thymidine before harvest. The relative rate of lymphoproliferation was calculated as the fold thymidine incorporation into cellular DNA over medium control (stimulation index).

Detection of epitope-specific $CD3^+CD8^+$ T lymphocytes by tetramer staining

Fresh PBMCs were stained with anti-human CD3 and CD8α Abs (BD Pharmingen) and Mamu-A*01 tetrameric complexes (provided by Dr. J. Altman, Emory University Vaccine Center at Yerkes, Atlanta, GA) refolded in the presence of a specific peptide and conjugated to PE-labeled streptavidin (Molecular Probes). $Gag_{181-189}$ CM9 (CTPYDINQM; Gag_CM9) and Tat_{28-35}SL8 (TTPESANL; Tat_SL8)-specific tetramers were used. Samples were analyzed on FACSCalibur (BD Biosciences), and the data are presented as the percentage of tetramer-positive cells of all $CD3^+CD8^+$ lymphocytes.

Intracellular cytokine (ICC) staining

SIV-specific $CD4^+$ and $CD8^+$ T cell responses were detected using pools of 15-meric peptides overlapping by 11 amino acids covering entire Gag, Rev, Tat, and Nef proteins of SIV_{mac239}. Cells (1×10^6) in RPMI 1640 medium (containing 10% human serum and antibiotics) were incubated in the absence or the presence of a specific peptide pool at 2 μg/ml of each peptide for 1 h as previously described (61). Brefeldin A (Sigma-Aldrich) at a final concentration of 10 μg/ml was added, and the cells were incubated for an additional 5 h. The cells were washed, stained for the surface Ags CD3ϵ and CD8α, permeabilized by incubation in FACSPerm solution, and stained with anti-TNF-α-FITC, and anti-IFN-γ-FITC Abs (all reagents and Abs from BD Pharmingen). The results were calculated as the total number of cytokine-positive cells with background subtracted.

Statistical analysis

All reported p values are two-sided. All correlation coefficients were calculated using the Spearman rank-order correlation test with a 95% confidence interval. Viral loads were compared by the Mann-Whitney rank-sum test. The Number Cruncher Statistical System (NCSS) and Sigmastat (version 2.0; SPSS) statistical software packages were used for the analyses.

[3] Abbreviations used in this paper: gpe, Gag-Pol-Env; ICC, intracellular cytokine staining; LPR, lymphoproliferative response; rtn, Retanef.

Results

Addition of immunogens encoded by early regulatory genes decreases the level of acute viremia and increases protection from simian AIDS

To assess the effect of immunization with early regulatory proteins, we have generated plasmid DNA and NYVAC-based vaccines expressing a chimeric polyprotein Retanef comprising genetically modified, attenuated, and reassorted SIV Rev, Tat, and Nef (DNA-*rtn* and NYVAC-SIV-*rtn*) (55) and directly assessed the effect of immunization with the chimeric Retanef vaccine alone or in combination with the Gag-Pol-Env (gpe) vaccine (8, 9). Twelve animals in the control groups were either not immunized (group 1A, four animals) or immunized with mock NYVAC (group 1B, eight animals; Fig. 1). DNA immunizations were given at weeks 0, 4, and 12 to animals in groups 2, 3, and 4; boosts with NYVAC-SIV vaccine were given at weeks 24 and 52 (Fig. 1). Animals in group 2 were immunized with the Retanef vaccine, animals in group 3 received both Retanef and gpe vaccines, and animals in group 4 received only the gpe vaccine. All animals were exposed to intrarectal challenge with 30 mucosal infectious doses of SIV_{mac251} virus during the memory phase of the immune response (6 mo after the last immunization). The immunization regimen of DNA plus recombinant NYVAC-SIV stock and the challenge virus used in all groups were the same to allow for a direct comparison. The results of the challenge of animals in groups 1B and 4 were described previously (9) and demonstrated that immunization with Gag, Pol, and Env provided a significant virological benefit during acute and chronic infection (Fig. 2*A*).

After intrarectal challenge exposure with SIV_{mac251}, the animals immunized with Retanef alone (group 2) displayed a slight delay in the onset of blood viremia (Fig. 2, *B–D*) and a statistically significant decrease in acute viremia compared with control animals ($p = 0.023$; Fig. 3*A* and Table I). Two of the macaques immunized with Retanef alone controlled viremia below 10^4 copies/ml plasma and maintained normal levels of $CD4^+$ T cell counts, whereas the remaining six animals experienced high viremia and died of AIDS within 32 mo (Fig. 2*B*, *middle panel*).

Importantly, immunization with Retanef in conjunction with gpe (group 3) resulted in a 3- to 7-day delay in the onset of viremia (Fig. 2, *B* and *C*) and a marked decrease in acute viremia compared with controls ($p < 0.001$), group 2 ($p < 0.001$), or group 4 ($p = 0.028$). Five of eight animals in this group contained virus below the level of detection or displayed a 2 $\log_{10}$ reduction of peak acute viremia. In two animals (no. 823 and 3165), virus was mostly contained at <500 RNA copies/ml plasma during the acute and chronic phases of infection (Fig. 2*B*, *lower panel*). These animals, however, were infected, because SIV DNA was detected in PBMCs (data not shown), and occasional "blips" of viremia were observed (animal 3165 at week 53 and animal 823 at week 70; Fig. 2*B*, *lower panel*). The peak of viremia in the remaining three animals appeared sharper and declined earlier than in controls.

The level of chronic viremia in group 3 was significantly lower than that in the controls ($p = 0.004$). Following the set point of viremia, five of eight immunized animals in group 3 maintained plasma levels at $<10^4$ copies/ml, whereas only one of 12 control animals did so (Fig. 3*B*). Four of eight animals in group 3 remained healthy 32 mo after challenge exposure (Fig. 3*C*). Three of them (animals 692, 823, and 3165) maintained normal $CD4^+$ T cell counts and low virus levels, whereas animal 822 experienced a progressive decline in $CD4^+$ T cells (data not shown).

Collectively, the results demonstrate that the addition of early regulatory genes to the structural genes in the vaccine resulted in a delay and significant reduction of acute viremia. The difference between groups 3 and 4 in their abilities to control virus during the chronic phase of infection did not reach statistical significance, however, these data are probably skewed by the presence of a higher number of Mamu-A*01 animals in group 4 compared with group 3 (five vs two; Table II). The Mamu-A*01 allele has been shown to provide advantage for long-term control of viral replication (58, 59, 62).

Evidence of Ag competition after vaccination and challenge with SIV_{mac251}

The design of these studies provided the opportunity to assess whether combining multiple Ags would result in Ag competition. At first we focused on the dominant epitope Gag_CM9 recognized by Mamu-A*01-positive animals. Immunization with SIV-*gpe*

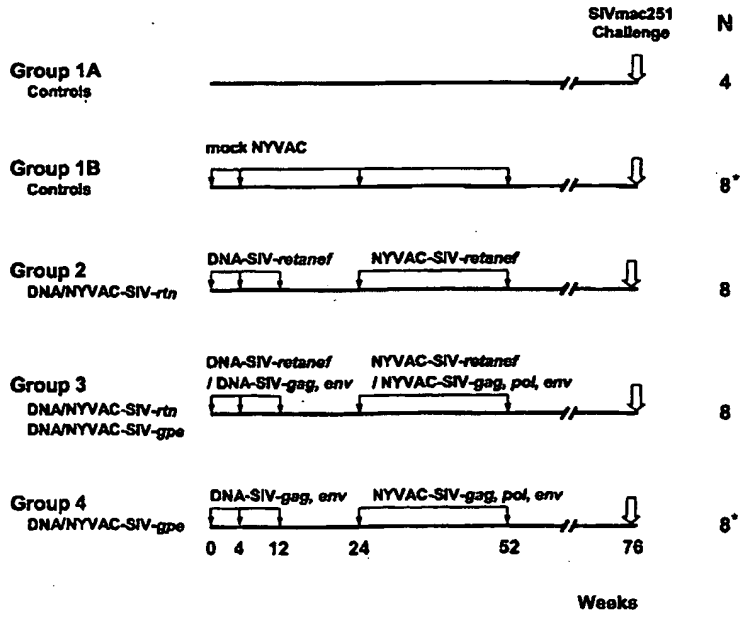

FIGURE 1. Schematic representation of the study design. The animals were immunized and challenged as described in *Materials and Methods*. N, number of animals included in each group. Data describing the immunization and challenge of animals in groups 1B and 4 (*) were published previously (8, 9) and are used for comparison purposes in this study.

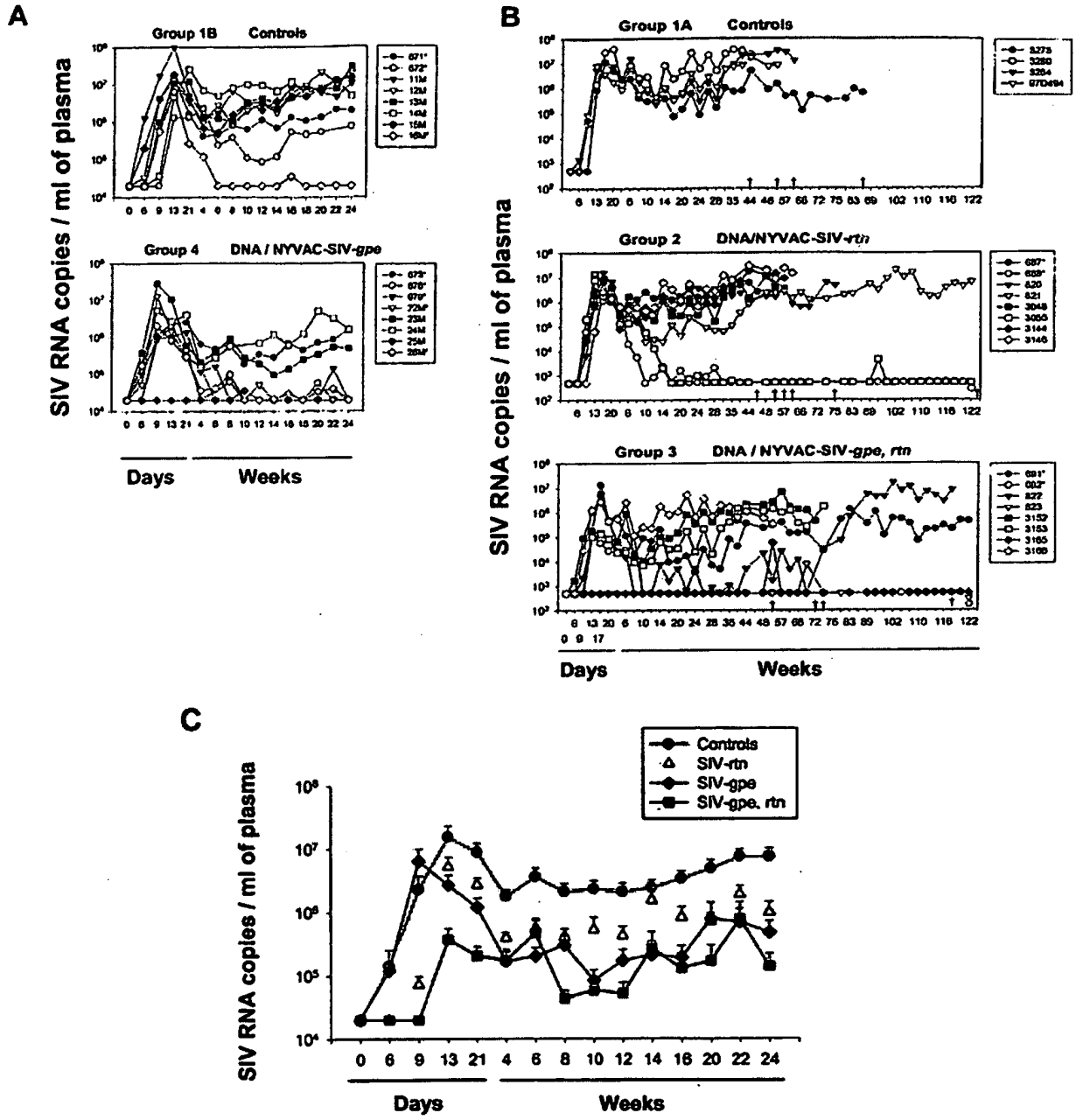

FIGURE 2. Virological outcome of SIV_{mac251} challenge exposure. *A* and *B*, Plasma viral levels in macaques after challenge exposure to SIV_{mac251}. *, Mamu-A*01-positive macaques; †, animal was euthanized due to severe health problems. *C*, Mean plasma levels of viral RNA in individual groups. Error bars indicate the SEs.

Ags induced higher responses in animals in group 4 than in macaques immunized with the combination of SIV-*gpe* and SIV-*rtn* vaccines (group 3; Fig. 4*A*).

Similarly, after the first boost with recombinant NYVAC, animals immunized with SIV-*gpe* alone displayed Gag- and Env-specific proliferative responses of higher peak magnitudes than those observed in animals vaccinated with SIV-*gpe* together with SIV-*rtn* (Fig. 4*B*). Consistent with these data, proliferative responses to Nef Ag were higher in macaques immunized with Retanef only than in those immunized with multigenic vaccines after the first recombinant NYVAC boost ($p = 0.007$; Fig. 4*B*). These intergroup differences were not evident after the second NYVAC boost, with the exception of Tat lymphoproliferative responses (LPRs), which were higher in group 2 than in group 3 ($p = 0.05$; weeks 53–56). Collectively, these data suggest that the combination of several immunogens decreases the relative frequency of specific T cells for a given Ag. This difference was evident and reached statistical significance at the time of maximal expansion of the immune response induced by the vaccines.

After mucosal exposure to SIV_{mac251}, all animals became infected. Mamu-A*01 animals developed robust responses to both immunodominant SIV epitopes Gag_CM9 and Tat_SL8 (Fig. 5*A*).

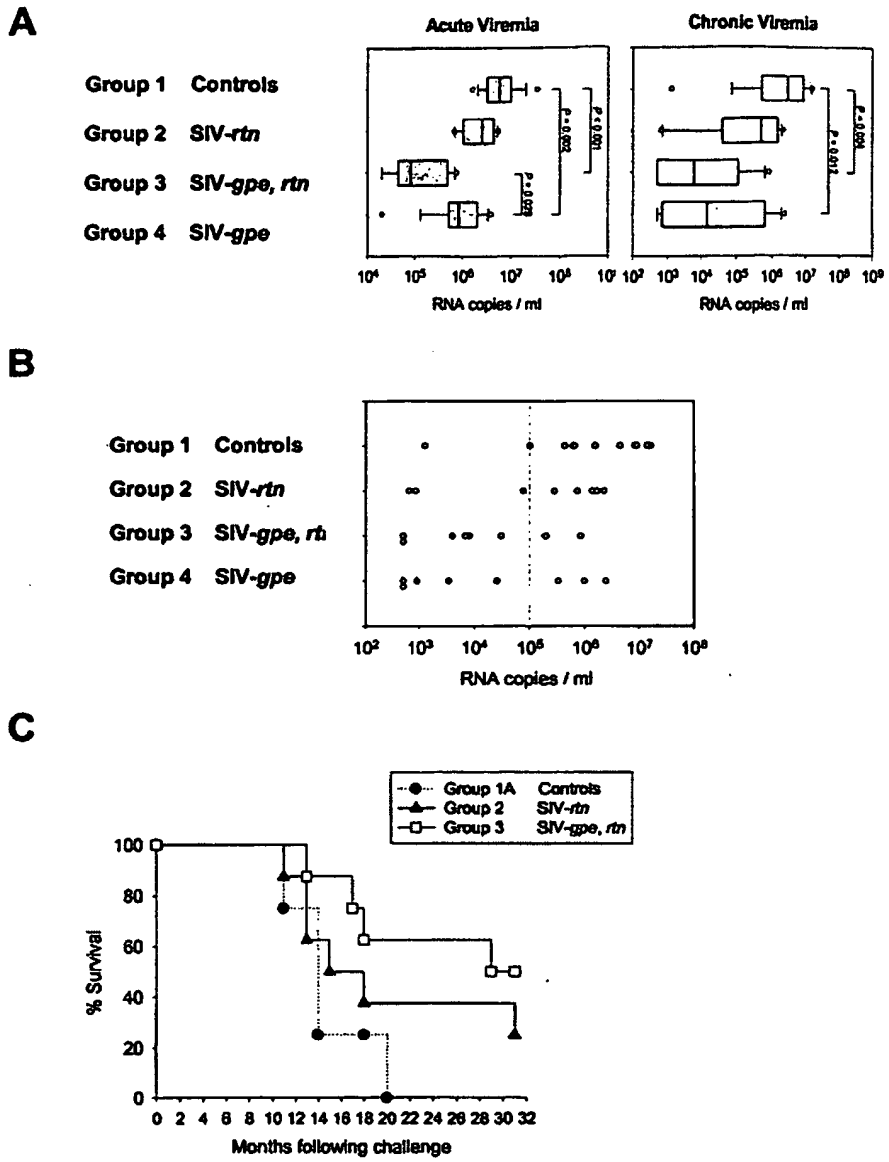

FIGURE 3. Clinical outcome of vaccination. *A*, Comparison of mean plasma viral levels in macaques from all groups during the acute (days 13–28) and post-set point chronic (weeks 16–24) phases of infection. Box plots represent the median and 25th, 75th, and 90th percentiles. The *p* values are based on two-tailed Mann-Whitney rank-sum test. *B*, Post-set point mean plasma viral levels (weeks 16–24) in individual animals. *C*, Survival curve analysis for animals in groups 1A, 2, and 3.

Consistent with the finding of epitope competition, macaques immunized with SIV-*rtn* alone experienced a faster and larger expansion of Tat_SL8-specific $CD8^+$ T cells (Fig. 5*A*, day 21) than either control macaques or macaques immunized with SIV-*gpe*. Likewise, animals immunized with gpe only had higher frequency and faster kinetics of appearance of Gag-specific cells (day 13) than either controls or animals immunized with Retanef. A balanced and more muted anamnestic response to these Ags was observed in animals receiving both the gpe and Retanef vaccines (Fig. 5*A*). The data confirm and add support to the observation of Ag competition during both primary and anamnestic responses.

LPRs to Gag p27 in the first 2 mo of infection were significantly higher in SIV-*gpe*-immunized groups 3 and 4 than in controls ($p < 0.001$ and 0.007, respectively) or the SIV-*rtn* only-immunized group 2 ($p = 0.002$ and 0.02), suggesting that vaccination had primed these responses (Fig. 5*B*). Similarly, Tat LPRs were higher in groups 2 and 3 than in control group 1 ($p = 0.004$ for both; Fig. 5*C*) and were preserved better in macaques immunized with the vaccine combination.

Next, SIV-specific $CD4^+$ and $CD8^+$ T cell anamnestic responses in the infected macaques were determined by ICC of cells stimulated with pools of overlapping 15-meric peptides. Macaques immunized with gpe (groups 3 and 4) had significantly higher numbers of Gag-specific $CD4^+$ T cells ($p < 0.03$) and a trend for higher $CD8^+$ T cells compared with other groups, whereas macaques immunized with Retanef only displayed a trend for an increase in the Rev, Tat, and Nef responses (Fig. 5*D*). The responses in group 3 were balanced, without apparent polarization toward any Ag.

Gag-specific $CD4^+$ T cell responses after infection were higher in gpe-immunized groups 3 and 4 compared with controls or group 2, demonstrating a classical recall response to Gag (Fig. 5*B*). Similarly, the frequencies of Rev-, Tat-, and Nef-specific $CD4^+$ T cells

Table I. *Statistical analysis of the levels of viremia in experimental groups during the acute and chronic phase of infection*[a]

		Chronic Viremia			
	Group	1	2	3	4
Acute Viremia	1			1 > 3 $p = 0.004$	1 > 4 $p = 0.012$
	2	1 > 2 $p = 0.023$			
	3	1 > 3 $p < 0.001$	2 > 3 $p < 0.001$		
	4	1 > 4 $p = 0.002$		4 > 3 $p = 0.028$	

[a] Values of p are based on two-tailed Mann-Whitney rank sum test. Acute viremia is defined as the average viremia at days 13, 20, and 28; chronic viremia is the average viremia at weeks 16, 20, and 24 postchallenge.

were higher in animals immunized with the Retanef only, suggesting an anamnestic response to early regulatory genes (Fig. 5*D*). The anamnestic responses with all these specificities were more variable in macaques from group 3 that had been immunized with a combination of gpe and Retanef vaccines.

Virological and immunological correlates of long-term containment of viral replication

The availability of virological and immunological data on a total of 24 immunized animals and 12 control animals provided the opportunity to assess the relationship between early and late virus levels as well as how the immune response induced by vaccination affected the virological outcome. We observed a direct correlation between the mean plasma virus RNA levels during primary infection and the mean plasma virus level after the set point (Fig. 6*A*), providing support for the concept that T cell-based vaccines that curtail early in infection are able to alter viral replication and delay the disease course. We have previously shown that preservation of Gag-specific $CD4^+$ T cells is important in determining the long-term virological outcome (8, 9, 63). We therefore analyzed a total

Table II. *MHC haplotypes of rhesus macaques used in the study*

	A01	A02	A08	A11	B01	B03	B04	B17
Group 1A: controls								
3275	−	−	−	−	−	−	−	−
3280	−	−	−	−	−	−	−	−
3284	−	−	−	−	−	−	−	−
97D474	−	−	−	−	−	−	−	−
Group 1B: controls								
671	+	−	−	−	+	−	−	−
672	+	−	−	−	−	−	−	−
11M	−	+	+	−	−	+	−	−
12M	−	+	−	−	−	−	−	−
13M	−	+	+	−	+	+	−	−
14M	−	−	−	−	+	−	−	−
15M	−	−	−	−	+	−	−	−
16M	+	−	−	−	−	−	−	−
Group 2: SIV-*rtn*								
687	+	−	−	−	+	−	−	−
688	+	+	−	−	−	−	−	−
820	−	−	+	−	+	−	−	+
821	−	+	−	−	−	−	−	−
3048	−	−	−	−	+	−	−	−
3055	−	−	−	−	+	−	−	−
3144	−	−	−	−	+	−	−	−
3146	−	−	−	−	+	−	−	−
Group 3: SIV-*gpe, rtn*								
691	+	−	−	−	+	−	−	−
692	+	−	−	−	−	−	−	−
822	−	−	−	−	−	−	−	−
823	−	−	+	−	−	−	−	−
3152	−	−	+	−	−	−	−	−
3153	−	−	−	−	−	−	−	−
3165	−	−	+	−	−	−	−	−
3166	−	−	+	−	−	−	−	−
Group 4: SIV-*gpe*								
673	+	−	−	−	−	−	−	−
678	+	−	−	−	+	+	−	−
679	+	−	−	−	+	−	−	−
22M	+	−	−	−	+	+	−	−
23M	−	+	−	−	+	−	−	−
24M	−	−	+	+	+	−	−	−
25M	−	+	−	−	+	−	−	−
26M	+	−	−	−	+	−	−	−

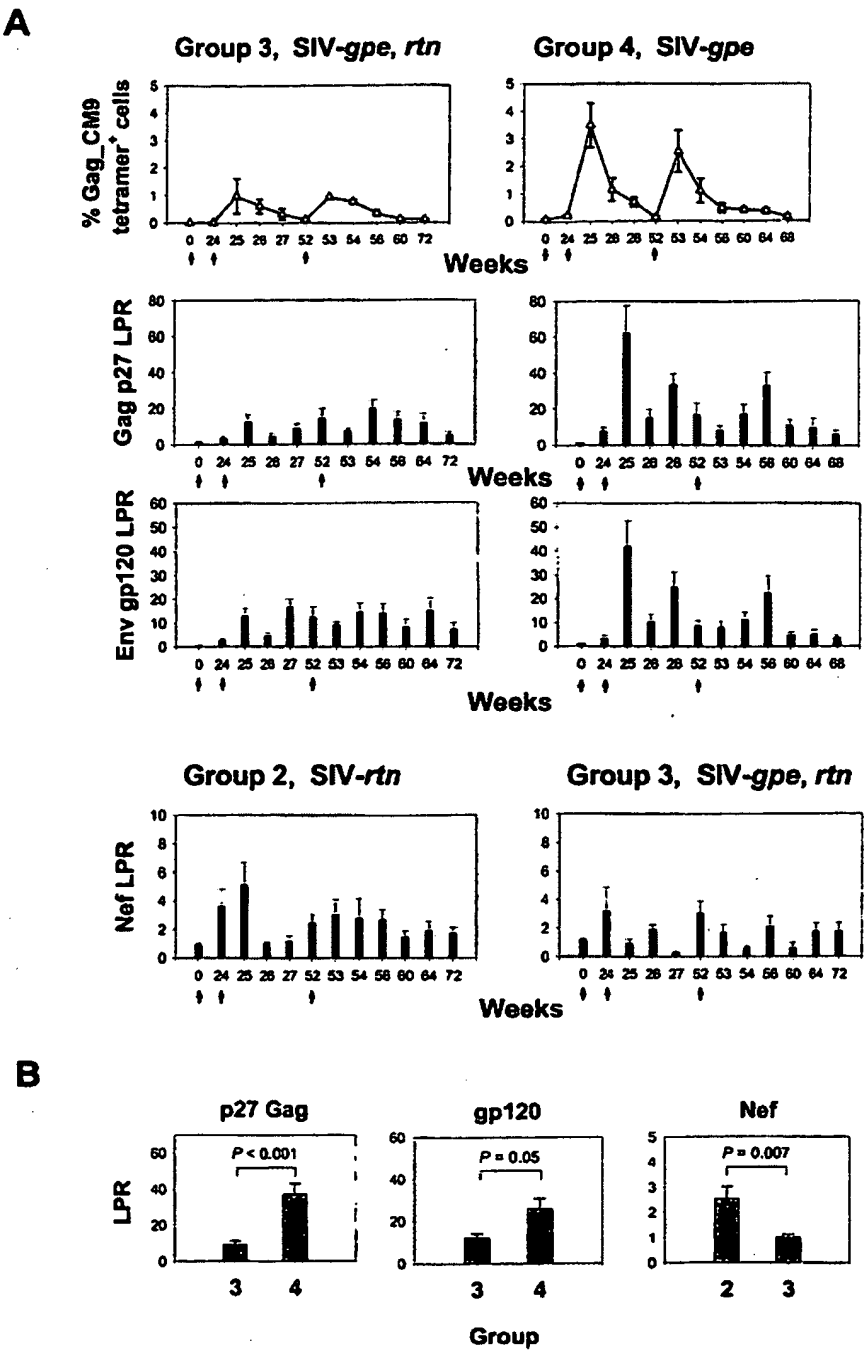

FIGURE 4. Immune responses induced by vaccination or SIV_{mac251} exposure. *A, Top panels,* Percentages of Gag_CM9-specific cells of the total $CD3^+CD8^+$ T cells over time as detected by tetramer staining of PBMCs in Gag-immunized groups 3 and 4. Background staining in Gag nonimmunized groups 1 and 2 was typically <0.1% (55) (data not shown). *Lower panels,* Proliferative responses to Gag p27, Env gp120, Nef, and Tat proteins. Values depict stimulation indexes over treatment with medium alone. Background levels in animals not immunized with relevant Ags were typically <2. Arrows indicate immunization at weeks 0, 24, and 52. Error bars indicate the SEs. *B,* Evidence of Ag competition after the first immunization with recombinant NYVAC. Mean LPR responses to p27 Gag, gp120 Env, and Nef proteins were detected during the 4 wk after the recombinant NYVAC immunization at week 24. Error bars represent the SEs. The p values were determined using the Mann-Whitney rank-sum test. Error bars indicate the SEs.

of 29 macaques for the frequency of Gag-specific $CD4^+$ T cells by ICC within a few weeks after infection and confirmed an inverse correlation with both acute and post-set point viremia levels ($p < 0.001$; Fig. 6*B*).

Confirming this observation, the LPRs to Gag Ag throughout the first 2 mo of infection also inversely correlated with both acute and post-set point viremia (Fig. 6*C*); LPR to Tat correlated with acute ($p = 0.01$), but not post-set point viremia (data not shown). In contrast, the inverse correlation between the numbers of Gag-specific $CD8^+$ T cells and acute or chronic viremia was weak and reached significance only on day 20 (Fig. 6*D*), but not on day 13 or at any other time after challenge. Together, these data suggest that the reduction of primary viremia results in a better preservation of Gag-specific $CD4^+$ T cell responses, which, in turn, may contribute to the maintenance of $CD8^+$ effector T cell function.

Discussion

Previously, we have demonstrated that immunization with SIV gpe in a DNA prime/poxvirus boost regimen confers significant suppression of viremia after mucosal challenge with highly pathogenic SIV_{mac251} (9). This study provides direct evidence of the benefit of addition of early regulatory proteins, Rev, Tat, and Nef, to the vaccine regimen. Immunization with a combination of early and structural/enzymatic proteins resulted in a 3- to 7-day delay in

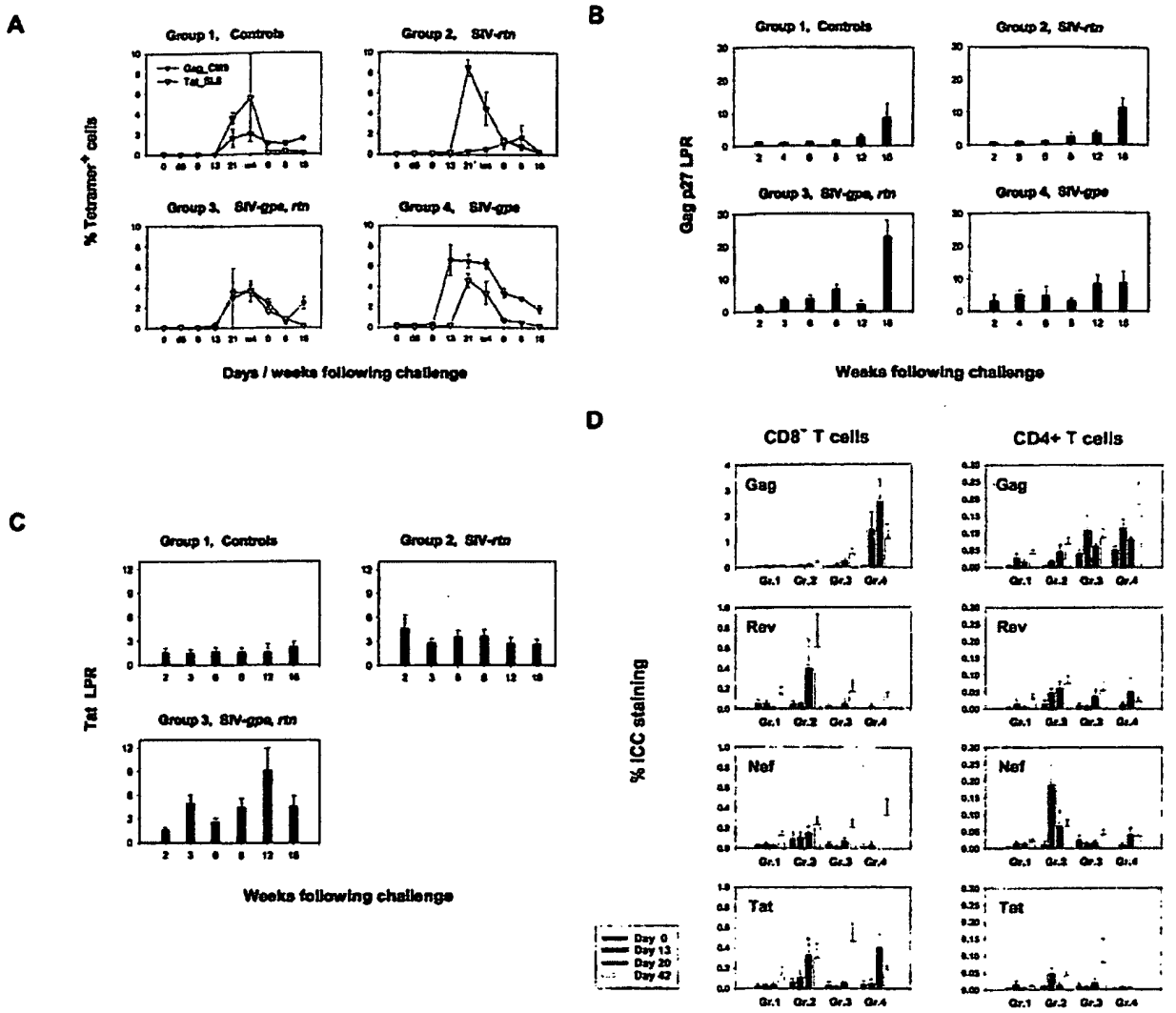

FIGURE 5. Immune responses after the challenge exposure to SIV_{mac251}. *A*, Postchallenge T cell responses to Gag_CM9 and Tat_SL8 epitopes as measured by tetramer staining of PBMCs in all groups. Error bars indicate the SEs. *B* and *C*, Postchallenge LPRs to Gag p27 and Tat proteins. Error bars indicate the SEs. *D*, Relative percentages of blood T cells specific for individual SIV Ags detected by ICC staining assay on days 0, 13, 20, and 42 after challenge with SIV_{mac251}. Group mean percentages of ICC^+ cells of total $CD3^+CD8^+$ or $CD3^+CD8^-$ ($CD4^+$) cells are shown; error bars represent the SEs.

acute infection, significant reduction of viral load in the initial phase of infection, lower set point virus levels, and increased survival. In contrast, vaccine based on regulatory SIV genes alone conferred only a slight delay and less significant reduction of acute infection and had little effect on the overall course of disease.

During the chronic phase of infection, virus levels did not differ significantly among the animals immunized with all Ags and those immunized only with gpe. We believe that this may be due to the presence of more animals in group 4 expressing Mamu-A*01 MHC allele (five of eight animals) compared with group 3 (two of eight), because the Mamu-A*01 molecule has been associated with natural resistance to SIV replication (58, 59). The underlying effect of given MHC class I haplotypes on disease progression was also addressed by screening macaque DNA for the eight most frequent Mamu alleles (Table II). With the exception of Mamu-A*01, no association between any particular Mamu allele and viral load was found.

The added beneficial effect of immunization with early proteins may be explained either by broadening immune responses or, alternatively, by special qualitative properties that distinguish these proteins from other viral Ags. CTL recognition of Nef, Rev, and Tat epitopes on cells in the early stage of infection results in their elimination before progeny virus is released (17, 21). In a series of elegant experiments, van Baalen et al. (22) have shown that CTL recognition of early, rather than late, Ags resulted in a $\geq$2- to 3-$\log_{10}$ reduction of virus production by similar numbers of effector cells in 10 days of culture. This qualitative difference between CTLs with specificity toward early vs late proteins may be especially important in the initial phase of infection in the absence of neutralizing Abs to Env (64) and could account for the delay of detectable virus in Retanef-immunized groups 2 and 3. Similar reduction and/or delay of acute viremia were observed in previous studies assessing the effect of immunization with early genes (20, 34, 43, 65). It should be noted that the level of the chimeric Retanef Ag used in this study was relatively low, at least as evidenced by the low levels of $CD8^+$ T cells induced against the immunodominant Tat_SL8 epitopes during vaccination, which contrast with the high levels of this response after infection. Thus, the

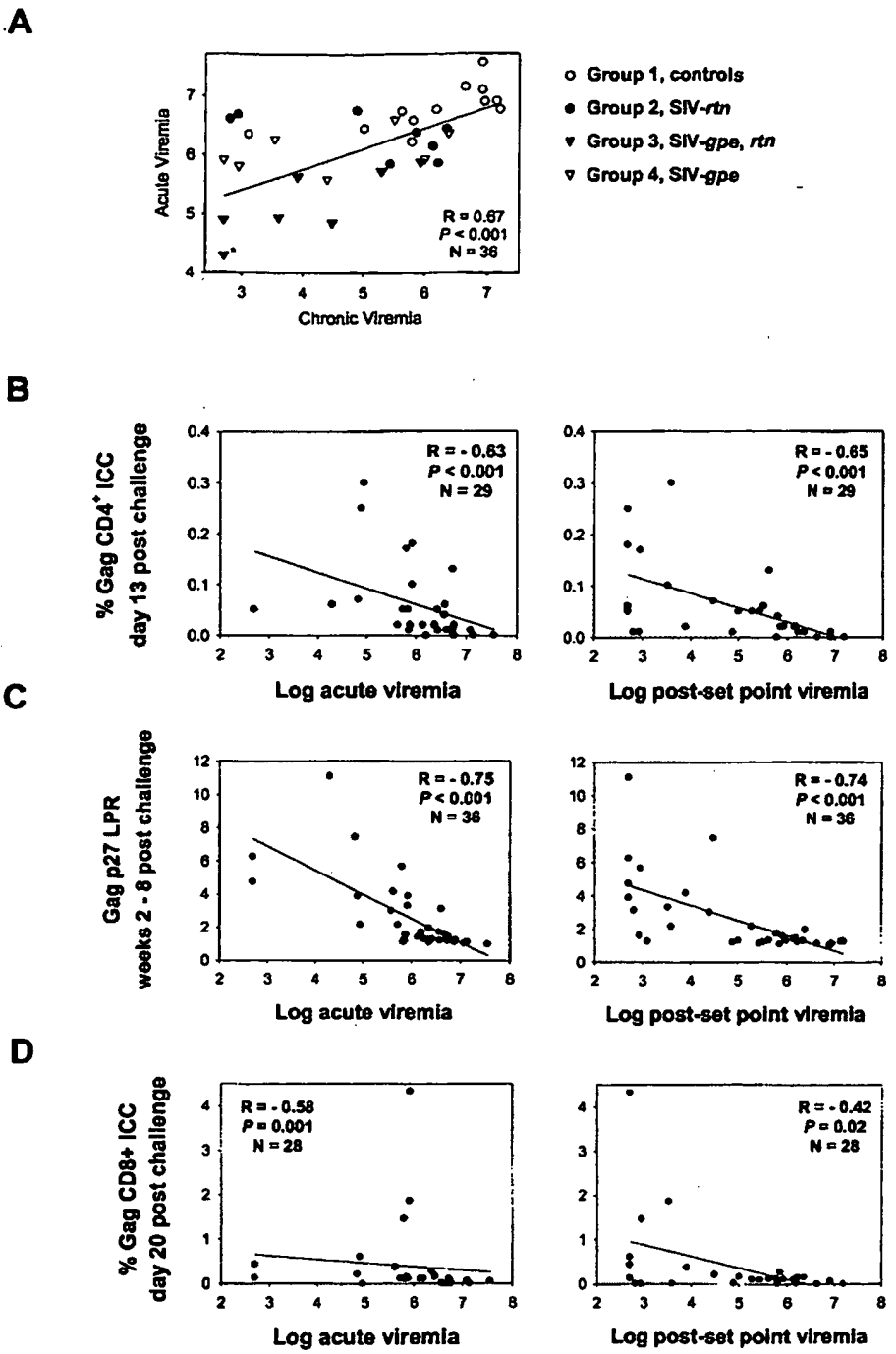

FIGURE 6. Virological and immunological correlates. Inverse correlation between the levels of acute (days 13–28) and post-set point (weeks 16–24) viremia in all 36 macaques studied (*A*). The *p* value was determined by Spearman rank-order correlation of data in 36 animals. *, Overlapping data from three animals. *B*, Correlation between the levels of acute (days 13–28) and post-set point (weeks 16–24) viremia and Gag-specific $CD4^+$ T cell responses detected on day 13 after challenge. *C*, Average Gag-specific LPRs (stimulation index) detected at weeks 2–8 after infection. *D*, Gag-specific $CD8^+$ T cell responses on day 20 after challenge. Correlation coefficients (*R*) and *p* values were determined by Spearman rank-order correlation test. Data from 29 of the 36 animals are presented.

beneficial effect of immunization with early viral proteins may be enhanced by using improved immunogens.

In this study we demonstrate that the reduction of the extent of acute SIV viremia has important implications for disease progression, because the level of acute viremia predicts the long-term course of infection (Fig. 6*A*). This is in line with previous observations (66). It has recently been demonstrated that a massive initial infection of memory $CD4^+$ T cells results in a depletion of 30–90% of these cells from the lymphoid tissues and mucosal surface during the first 2 wk of infection (1–3). The early destruction of the $CD4^+$ memory compartment represents an initial insult from which the organism may never recover and which may be the distinguishing feature setting HIV apart from chronic infections with other nonlytic viruses (5). The loss of $CD4^+$ T cell regulatory and effector functions, especially at the tissue-environmental interfaces, may increase the vulnerability to pathogens, contribute to chronic immune hyperactivation, and contribute to the destruction of lymphoid tissue. Our results support the idea that rapid recruitment of pre-existing vaccine-induced T cells specific for early Ags may be important in the

initial suppression of virus growth and preservation of memory $CD4^+$ T cell function.

There are several lines of evidence that support this hypothesis. HIV-specific $CD4^+$ T cells that maintain proliferative capacity are detected in long-term nonprogressors and patients treated with highly active antiretroviral therapy; however, they are drastically reduced in number and/or function in individuals with high levels of plasma HIV (67, 68). These cells were shown to be a preferential target of HIV (69), an observation not surprising given their spatial and temporal colocalizations with HIV-producing cells in the local microenvironment of infected lymphoid tissue. We and others (63, 68, 70–72) have demonstrated that early suppression of acute viremia with antiretrovirals results in a preservation of virus-specific $CD4^+$ T cell responses and long-term control of infection. In this study we confirm and extend our previous observation that the ability to control infection directly correlates with the preservation of Gag-specific $CD4^+$ T cells and their proliferative capacity during primary viremia (Fig. 6, *B* and *C*). These data go hand-in-hand with the idea that early expansion of vaccine-induced virus-specific T cells may result in the preservation of pre-existing and/or newly recruited virus-specific $CD4^+$ T cells.

Although the immunization with early proteins alone delayed the onset of viremia, the benefit was rapidly lost, probably due to virus escape from immune recognition (23). Accumulated evidence suggests that more stable epitopes, such that the virus cannot escape without a significant toll on its fitness, are needed for long-term maintenance of protection (73–75). This could explain our observation that a combination of early and structural genes improved the overall control of viral replication.

The data presented in this study are germane to the ongoing debate on whether to include early viral proteins as part of an HIV vaccine. Among the arguments used against the inclusion of these proteins in a vaccine is that their sequence is highly variable at each amino acid position, and only a few conserved regions can be identified. A second argument stems from the fact that presentation of these early Ags in the acute phase of infection may focus the immune response toward epitopes that can readily escape without affecting viral fitness. Thus, early recognition of these epitopes may divert responses to more conserved and protective epitopes, the mutation of which affects viral fitness (72, 73). Our data suggest that an effective HIV vaccine should induce broad responses to both types of epitopes. Despite their transient character, the responses directed to the epitopes that are subject to variation without significant loss of viral fitness, such as those found in early regulatory proteins, may still wield a significant effect on the control of virus proliferation during the decisive initial days of infection. The Retanef Ag used in this study constitutes only 16% of the protein sequence of all Ags used in the complex vaccine, suggesting that the delay and lower level of acute viremia may be due to qualitative differences in immune responses to the respective Ags.

An important aspect of this study is the observation of Ag competition, manifested by a relative reduction of $CD8^+$ T cell responses to Gag and Tat and LPRs to Gag, Env, Tat, and Nef after immunization with combined vaccines. This reduction probably results from competition for a common niche and reflects possible size limits of effector $CD8^+$ and $CD4^+$ T cell populations (76–78). The competition is probably accentuated by a concomitant induction of T cells specific for the vaccinia virus backbone (79). The dominance of secondary virus-specific responses after the second boost with recombinant NYVAC vectors could explain the lower overall responses to SIV Ags as well as the apparently lesser competition between them. Possibly, staggering immunization temporally and spatially with DNA and recombinant poxviruses expressing diverse Ags will improve vaccine immunogenicity and relative efficacy.

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Disclosures

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Safety and Immunogenicity of ALVAC vCP1452 and Recombinant gp160 in Newly Human Immunodeficiency Virus Type 1-Infected Patients Treated with Prolonged Highly Active Antiretroviral Therapy

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In order to boost immune responses in persons in whom highly active antiretroviral therapy (HAART) was initiated within 120 days of the onset of symptoms of newly acquired human immunodeficiency virus type 1 (HIV-1) infection, we administered vaccines containing a canarypox virus vector, vCP1452, with HIV-1 genes encoding multiple HIV-1 proteins, and recombinant gp160. Fifteen HIV-1-infected subjects who achieved sustained suppression of plasma viremia for at least 2 years were enrolled. While continuing antiretroviral therapy, each subject received at least four intramuscular injections of the vaccines on days 0, 30, 90, and 180. Adverse events were mild, with the most common being transient tenderness at the vCP1452 injection site. Of the 14 patients who completed vaccination, 13 had significant increases in anti-gp120 or anti-p24 antibody titers, and 9 had transient augmentation of their T-cell proliferation responses to gp160 and/or p24. HIV-1-specific $CD8^+$ T cells were quantified using an intracellular gamma interferon staining assay. Among 11 patients who had increased $CD8^+$ T-cell responses, seven had responses to more than one HIV-1 antigen. In summary, vaccination with vCP1452 and recombinant gp160 appears safe and immunogenic in newly HIV-1-infected patients on HAART.

Administering highly active antiretroviral therapy (HAART) to human immunodeficiency virus type 1 (HIV-1)-infected individuals results in a rapid, sustained, and highly significant reduction of plasma viremia in most patients (23, 58). The virologic and immunologic consequences of HAART have resulted in a dramatic reduction in HIV-1 infection-related morbidity and mortality (39). However, the existence of latently infected resting memory $CD4^+$ T cells has made the eradication of HIV-1 infection with HAART alone problematic (6, 15, 59). The goal of eradication may be even more difficult to attain due to the presence of residual viral replication during therapy (14, 19, 41, 61). As a result, irrespective of the time of initiation of therapy, cessation of HAART is accompanied by a rebound in viremia in days to weeks in most if not all treated patients (11, 20, 21, 36).

These findings are clear indicators that current HAART regimens alone are unable to reduce total body viral burden to levels controllable by host immune responses in the absence of drug. Given the long-term toxicities of HIV-1 therapies, the risk of the emergence of drug resistance, and the cost of lifelong HAART, the need to define treatment strategies to limit drug exposure has become critical.

To achieve durable viral suppression after a finite course of HAART, alternative treatment strategies are needed. Several lines of evidence suggest that strong cellular immune responses contribute to the control of retroviral replication in the absence of antiretroviral treatment (5, 24, 27, 37, 44, 47, 49). Therefore, we hypothesized that the use of adjunctive vaccination, if capable of augmenting HIV-1-specific immune responses, may provide a beneficial virologic outcome in HIV-1-infected persons treated with HAART who elect to discontinue therapy.

Studies suggest that an effective HIV-1 vaccine, either therapeutic or preventative, should stimulate broadly reactive humoral and cellular immunity, in particular cytotoxic T-lymphocyte (CTL) responses. A number of experimental vaccines have conferred protective immunity against intracellular pathogens, such as malaria, by stimulating strong immune responses in animal models (50, 52). Vaccine strategies directed against HIV-1 include the use of recombinant proteins, peptides, recombinant bacterial or viral vectors, and DNA (9; NIH AIDS Vaccine Evaluation Group, posting date 9 September 1999).

Recombinant protein and peptide vaccines are single-component vaccines that stimulate either humoral or cellular immune responses, but not both, and thus are not ideal candidates in a therapeutic setting. The bacterial vectors and DNA vaccines in development were not available for use in seronegative or seropositive individuals when we initiated this trial.

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TABLE 1. Patient characteristics

Subject no.	Time from onset of symptoms to treatment (days)	Baseline findings		Treatment[a]	Duration of HAART (days)	HLA types					
		HIV-1 RNA (log_{10})	$CD4^+$ T cells (cells/mm^3)			A1	A2	B1	B2	C1	C2
313-2	90	3.9	564	A	1,182	2	2	15	39	3	12
313-7	90	5.0	534	A	1,070	33	70	14	56	3	8
900	120	4.1	609	D	974	1	4	7	9	7	7
904	60	4.4	689	D	1,066	11	23	15	59	3	3
905	60	5.1	954	D	927	3	31	13	36	6	8
908	80	4.4	307	D	962	16	69	39	50	7	7
918	9	6.2	1,121	D	867	1	33	8	36	4	8
921	30	5.6	311	D	825	1	1	8	8	7	7
1306	30	5.3	546	B	817	2	2	13	56	3	7
1308	9	6.2	432	B	1,038	2	32	40	42	2	18
1309	90	4.2	500	B	807	2	30	18	45	5	5
1310	120	4.0	387	B	1,020	3	24	56	58	7	19
2001	60	4.6	290	C	1,225	1	3	35	52	4	16
3002	60	5.0	459	C	992	1	2	15	38	3	7
Mean ± SD	65 ± 36	4.9 ± 0.8	550 ± 240		984 ± 129						

[a] A, zidovudine (ZDV), lamivudine (3TC), and ritonavir (RTV); B, ZDV/3TC/indinavir (IDV); C, ZDV/3TC/RTV/saquinavir; D, ZDV/3TC/abacavir/amprenavir.

However, at the time this trial was developed, limited studies using earlier versions of the recombinant canarypox viruses (ALVAC) in combination with HIV-1 envelope proteins had been performed. It had been demonstrated that this strategy was safe in uninfected (12) as well as HIV-1-infected individuals (40). Furthermore, these earlier constructs were also capable of generating some degree of humoral and cellular immunity in seronegative individuals (1, 7, 16).

The excellent safety record of ALVAC vaccines is attributed to their virologic properties. Canarypox viruses belong to the *Avipox* genus of the *Orthopoxviridae* family of DNA viruses. Although replicating well in avian cells, they do not replicate productively in mammalian cells (53–56). Despite a self-limiting abortive replication cycle, canarypox vaccine vectors can efficiently infect antigen-presenting cells and express proteins encoded by inserted genes under the control of early promoters (13). This may result in sufficient antigenic stimulation to elicit cellular immune responses.

Several generations of ALVAC vaccines have been designed and constructed over time to include single or multiple HIV-1 genes (35). The vector used in the current study, vCP1452, is a third-generation ALVAC vaccine, which includes not only multiple HIV-1 genes and a number of HLA-A2-restricted CTL epitopes from both the Pol and Nef regions of HIV-1, but also two vaccinia virus-derived antiapoptosis genes to enhance protein expression in mammalian cells. We therefore selected this recombinant vaccine to be the cornerstone of our vaccine strategy.

Here, we describe the first safety and immunogenicity study of the novel recombinant vCP1452 in combination with recombinant gp160 in 15 newly HIV-1-infected patients who were treated with HAART and achieved durable viral suppression for a minimum of 2 years. To determine levels of vaccine-induced $CD8^+$ T-cell-mediated HIV-1-specific immune responses, we developed an assay that used both the property of cytokine production from stimulated T cells and an objective gauging instrument, fluorescence-activated cell sorting (FACS), as a read-out. This assay is detailed below. We believe this pilot study establishes the scientific rationale for future investigations combining therapeutic vaccines and HAART as a strategy to treat HIV-1 infection.

MATERIALS AND METHODS

Patients. Individuals included in the vaccine study were diagnosed and treated within 120 days of the onset of symptoms of acute HIV-1 infection. Treatment included zidovudine and lamivudine in combination with ritonavir, indinavir, ritonavir/saquinavir, or abacavir/amprenavir (Table 1). Inclusion criteria required participants to have sustained levels of plasma HIV-1 RNA below the detection level of 50 copies/ml (less than one detectable HIV-1 RNA determination per treatment year). All vaccine recipients were born prior to 1972 (latest, 1967) and had received previous vaccination with vaccinia virus. Subjects were treated for 983 days on average (range, 807 to 1,225 days); their CD4 counts were 779 on average (range, 449 to 1,311/μl) (Table 1). In addition, their HLA-A, -B, and -C loci were determined by using sequence-specific primers according to published methods (10, 38) (Table 1). All studies were approved by the Rockefeller University Institutional Review Board and conducted in accordance with good clinical practices. Written informed consent was obtained from all study participants.

Vaccines and vaccination schedule. Each patient received at least four vaccinations at days 0, 30, 90, and 180. Each vaccination included 10^7 50% tissue culture infective doses of ALVAC vCP1452 and 50 μg of recombinant gp160 (rgp160), both kindly provided by Aventis Pasteur (Lyon, France). The recombinant canarypox virus ALVAC vCP1452 expresses the products of several HIV-1 genes, including gp120, expressed by a part of the *env* gene of the HIV-1 MN strain, and the anchoring transmembrane region of gp41 of the HIV-1 LAI strain; the p55 polyprotein expressed by the *gag* gene of the HIV-1 LAI strain: a portion of the *pol* gene sufficient to express protease activity from the HIV-1 LAI strain in order to process the $p55^{gag}$ polyprotein; and genes expressing peptides from *pol* and *nef* known to be HLA-A2-restricted cytotoxic T-cell lymphocyte epitopes. Two vaccinia virus-derived coding sequences are also incorporated in the recombinant virus to improve RNA translation and the expression of HIV-1 gene products.

The recombinant vaccine virus was grown on pathogen-free chicken embryo fibroblasts, and the vaccine was suspended in a solution of serum-free, antibiotic-free culture medium containing virus stabilizers and lyophilized. The recombinant gp160 is a hybrid glycoprotein consisting of gp120 from the HIV-1 strain MN and gp41 from the HIV-1 strain LAI.

Vaccines were given intramuscularly in the forearm (ALVAC on the left, rgp160 on the right). After each vaccination, patients were observed in the clinic for 30 min for potential side effects. In addition, either a physician or a nurse contacted all vaccine recipients within 72 h of each vaccination to document any adverse events. Subjects were followed weekly for 2 weeks following the first vaccine and 1 week after each subsequent vaccination. HAART was continued throughout the vaccination period. Patient diaries were also provided to record adverse events.

Virologic determinations. Longitudinal plasma HIV-1 RNA levels were measured using a reverse transcription PCR (Amplicor HIV-1 Monitor Ultra Sensitive; Roche Molecular Systems, Inc., Alameda, Calif.) that has a lower limit of detection of 50 HIV-1 RNA copies/ml.

Detection of antibody responses. Levels of plasma antibody to HIV-1 $gp120_{JRFL}$ (Progenics, Tarrytown, N.Y.) and $p24_{SF2}$ (Chiron, Emeryville, Calif.) proteins were detected using a standard enzyme-linked immunosorbent assay (ELISA) protocol as described previously (4).

Lymphocyte proliferation assay. The proliferative responses of peripheral blood mononuclear cells (PBMC) were measured using a standard [^{3}H]thymidine (DuPont NEN, Boston, Mass.) incorporation assay as described (48). The concentration of HIV-1 antigens used for stimulation was 5 μg/ml for both HIV-1 $gp160_{LAV}$ and $p24_{NY5}$ (Protein Sciences, Meriden, Conn.).

ICS. For intracellular cytokine staining, aliquots of 0.5×10^6 to 1×10^6 cryopreserved PBMC from patients were infected with recombinant vaccinia viruses expressing HIV-1 Env, Gag, Pol, Nef, or control Eco antigens (Virogenetics, Troy, N.Y.) at a multiplicity of infection (MOI) of 2.0 for 18 to 20 h at 37°C. Then 10 μg/ml of brefeldin A (Golgiplug; PharMingen, San Diego, Calif.) was added during the last 5 h of incubation. The cells were stained with anti-CD3PE, -CD4APC, and -CD8PerCP (Becton-Dickson, San Jose, Calif.) antibodies for 30 min at 4°C. After washing, cells were permeablized with CytoFix/Cytoperm solution (PharMingen, San Diego, Calif.), then stained intracellularly by an anti-gamma interferon (IFN-γ)-fluorescein isothiocyanate antibody (PharMingen, San Diego, Calif.) before being analyzed using a FACScalibur flow cytometer.

The FACS data were analyzed with CellQuest (Becton Dickinson, San Jose, Calif.) software by first gating on small $CD3^+$ T cells followed by analysis of the $CD8^+$- and IFN-γ-staining cell populations. The results were expressed as the percentage of $CD8^+$ T cells producing IFN-γ. In the initial 195 consecutive assays in 43 HIV-1-infected individuals, the negative control antigen Eco stimulated 0.02 ± 0.03% of $CD8^+$ T cells to produce IFN-γ, whereas 5 μg of of the positive control superantigen, staphylococcal enterotoxin B (SEB), per ml stimulated between 1 and 20% of the $CD8^+$ T cells to produce IFN-γ. Based on these preliminary experiments, 0.05% of IFN-γ-producing cells was determined to be significantly above the background and considered a positive value.

RESULTS

Safety of vCP1452. Fifteen subjects who satisfied the entry criteria and gave informed consent were consecutively enrolled from various studies of HAART during early and acute HIV-1 infection conducted at the Aaron Diamond AIDS Research Center. A total of 62 vaccinations were administered to 15 subjects. One subject was lost to follow-up after two vaccinations and was excluded from all subsequent analyses except for safety assessments. The vaccine recipients were mainly Caucasian males, infected sexually, and symptomatic for 9 to 120 days before initiating treatment.

On average, $CD4^+$ T cells increased by 246 cells/μl, and levels of HIV-1 RNA fell 3.2 logs to undetectable levels during a mean of 984 days of treatment with HAART (range, 807 to 1,225 days) (Table 1). Each patient received at least four vaccinations on days 0, 30, 90, and 180. ALVAC vCP1452 and rgp160 vaccines as described above were given intramuscularly in the left and right upper forearms, respectively. Two subjects received a fifth vaccination (313-2 and 1306) on day 270. Subject 313-7 received additional vaccinations on days 270 and 340. All vaccine recipients were given the option of continuing HAART with or without additional vaccinations at 90-day intervals as well as elective discontinuation of antiviral therapy at least 7 days following their fourth or final vaccination.

Transient tenderness in the left arm at the ALVAC 1452 injection site, without swelling or redness, was the most common adverse event reported, occurred in 36 of 62 (58%) vaccinations, and was reported at least once in all 15 subjects. One episode of low-grade fever between 99 and 100°F occurred in 4 of 62 (6.4%) vaccinations in four subjects. Mild transient headache occurred in 4 of 62 vaccinations in four subjects. Headache and low-grade fever elevation did not occur concurrently. All adverse events were mild and of short duration and resolved either without therapy or after treatment with ibuprofen or acetaminophen.

Virologic parameters and levels of $CD4^+$ T cells changed minimally during vaccination. Prevaccination plasma viremia levels in all study subjects fell below the detection limit of 50 copies/ml within 6 months of initiating HAART. All subjects experienced less than one episode of measurable viremia per treatment year during the subsequent 20 to 40 months before receiving the first vaccination.

During vaccination, occasional episodes of low-level viremia were observed in four patients (900, 908, 1306, and 3002) (Fig. 1). Viremia in subject 900 was due to admitted nonadherence to the HAART regimen and occurred late in the course of vaccination. An unexplained period of plasma viremia of 100 copies or less appeared 4 weeks after subject 908 received the first vaccination. Despite receiving subsequent vaccines, plasma viremia returned to undetectable levels within 1 month and remained so for the duration of the vaccine study. Subject 3002 had low-level viremia temporally associated with the second vaccination. Subject 1306 had one isolated viremic episode of 85 copies/ml during the 3-month interval between the third and fourth vaccinations that was temporally unrelated to observed vaccine-induced immune responses.

There was no apparent detrimental effect on $CD4^+$ T-cell counts associated with vaccination. In fact, levels of $CD4^+$ T cells were generally maintained in all vaccine recipients (Fig. 1). There were no significant changes in lymphocyte subsets or markers of activation during the study period. Both $CD45RA^+$ and $-RO^+$ cells increased from a baseline of 261 and 491 cells to 329 and 542 cells/μl, respectively. The activation status of $CD4^+$ T cells, as measured by HLA-DR and Ki67 expression, varied little pre- and postvaccination (data not shown).

Vaccination boosted HIV-1 binding antibody production. The binding antibody responses to HIV-1 Gag-p24 and Env-gp120 were monitored longitudinally. All except one patient (921) had a 0.5 to 2 log increase in anti-gp120 antibody levels postvaccination. In addition, three patients (313-2, 313-7, and 2001) had a marked increase (1.0 ± 0.3 log) in anti-p24 response (Fig. 2, Table 2). The heightened anti-p24 response in these subjects was temporally associated with a significant augmentation of the T-cell proliferative response to the same antigen (Fig. 3). Of note, the antibody levels generally reached a plateau after the second or third vaccination (Fig. 2). Subject 921 not only failed to mount a humoral response to vaccination but exhibited an unexplained precipitous drop in the level of anti-gp120 antibody postvaccination (Fig. 2). Levels of antibody to gp120 in subject 918 increased more than 2.0 log shortly after the first vaccination but also exhibited a rapid decline. This individual did not develop a humoral response to subsequent vaccinations (Fig. 2).

To determine whether neutralizing antibodies to laboratory strains of HIV-1 were induced by vaccination, five plasma samples from two subjects (313-2 and 1309) were used in neutralization assays against a CCR-5-using virus, JR-CSF, and a CXCR-4-using virus, NL4-3. Neither subject exhibited

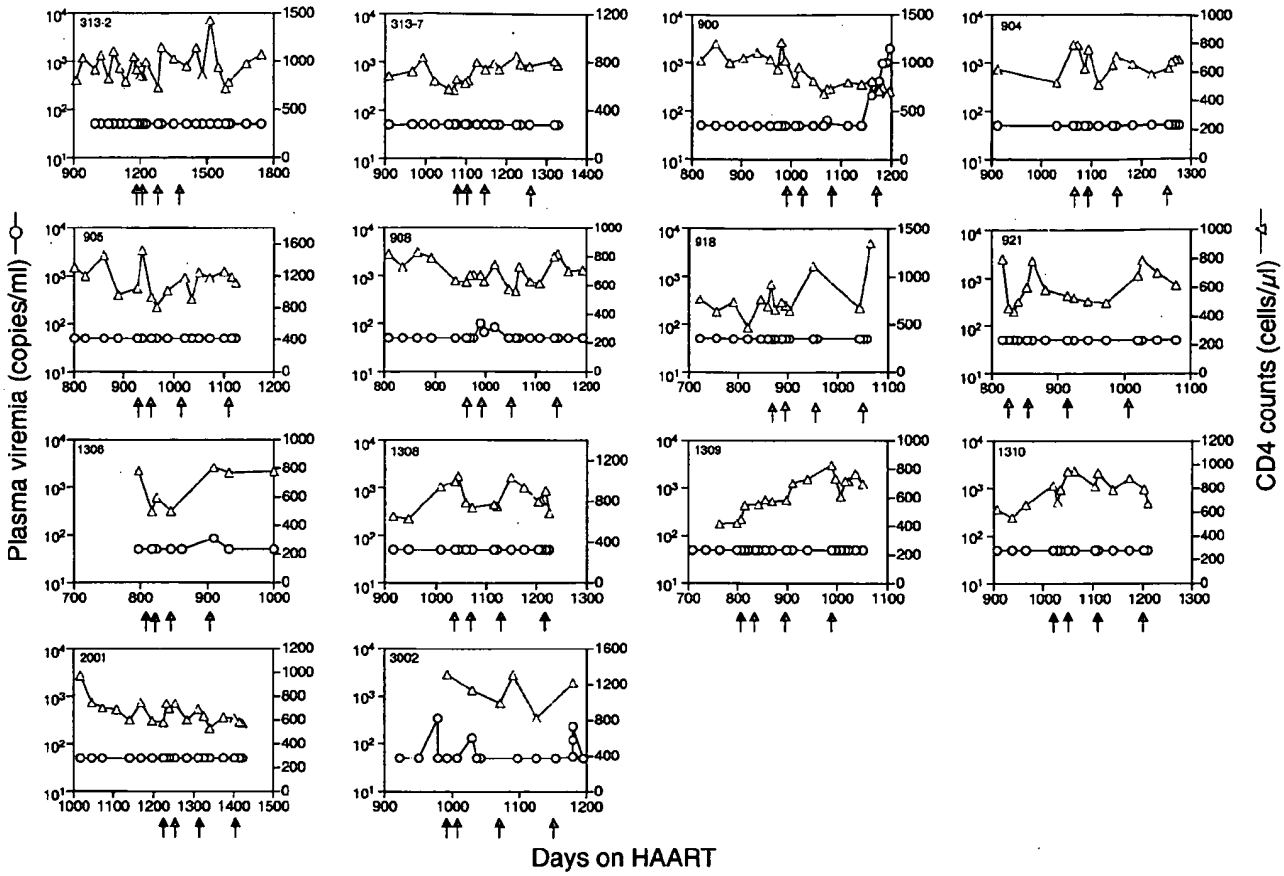


FIG. 1. Virological and immunological changes observed during vaccination. The plasma HIV-1 RNA levels (circles) and CD4 counts (triangles) of each patient were assessed longitudinally. Each panel represents results for one patient, and the arrows indicate days of vaccination.

neutralizing activity at any time point postvaccination (data not shown).

Transient T helper cell responses were induced by vaccination. HIV-1-specific T helper cell activity was examined using a standard lymphocyte proliferation assay with freshly isolated PBMC (48). Eight vaccinated subjects (313-2, 313-7, 900, 904, 908, 1308, 1309, and 3002) had early, transient elevations of anti-gp160 responses that returned to baseline levels soon after the initial peak. Gag-p24-specific T helper cell responses were briefly augmented in eight vaccine recipients (313-2, 313-7, 900, 905, 908, 1308, 1309, and 3002) (Fig. 3, Table 2). Seven of these eight patients also had Env-gp160-specific T-cell proliferative responses, and two of them (313-2 and 313-7) had strong anti-p24 antibody responses. Of note, the p24- and gp160-specific responses were boosted for a second time in three subjects (904, 1308, and 1309) with further vaccination, but were not sustained (Fig. 3).

Quantifying the number of virus-specific IFN-γ-producing $CD8^+$ T cells using intracellular cytokine staining. At the time of study initiation in 1998, we decided to explore the possibility of developing an assay that used both the property of cytokine production from stimulated T cells and an objective gauging instrument, FACS, as a read-out. This assay, called intracellular cytokine staining, is illustrated by the following example. Cryopreserved PBMC from an HIV-infected patient were stimulated with recombinant vaccinia virus expressing various HIV-1 or control antigens for 18 to 20 h at 37°C. Then 10 μg/ml BFA was added for the last 6 h of incubation. The cells were first stained with anti-CD3, -4, and -8 antibodies and then stained with an anti-IFN-γ antibody and subjected to analysis by FACS. During the analysis, we gate on the $CD3^+$ small lymphocyte and display a two-dimensional density plot of CD8 versus IFN-γ.

As shown in a typical experimental result (Fig. 4a), only 0.02% of $CD8^+$ T cells produced IFN-γ in response to stimulation with a non-HIV antigen (Eco). In the positive control samples stimulated with SEB, 9% of the total $CD8^+$ T cells produced IFN-γ. The range of IFN-γ produced by HIV-1 antigenic stimulation lies between the positive and negative controls. Env, Gag, and Pol/Nef stimulated 0.16%, 0.10%, and 0.58% $CD8^+$ T cells, respectively, while Pol stimulated minimal IFN-γ production in this patient (Fig. 4a).

To determine if this newly developed intracellular cytokine staining assay was suitable for the study of HIV-1-specific $CD8^+$ T-cell responses in HAART-treated patients, we performed this assay on samples from a large number of such individuals. We performed 195 assays for 43 patients and found that the negative control antigen Eco stimulated 0.02 ±

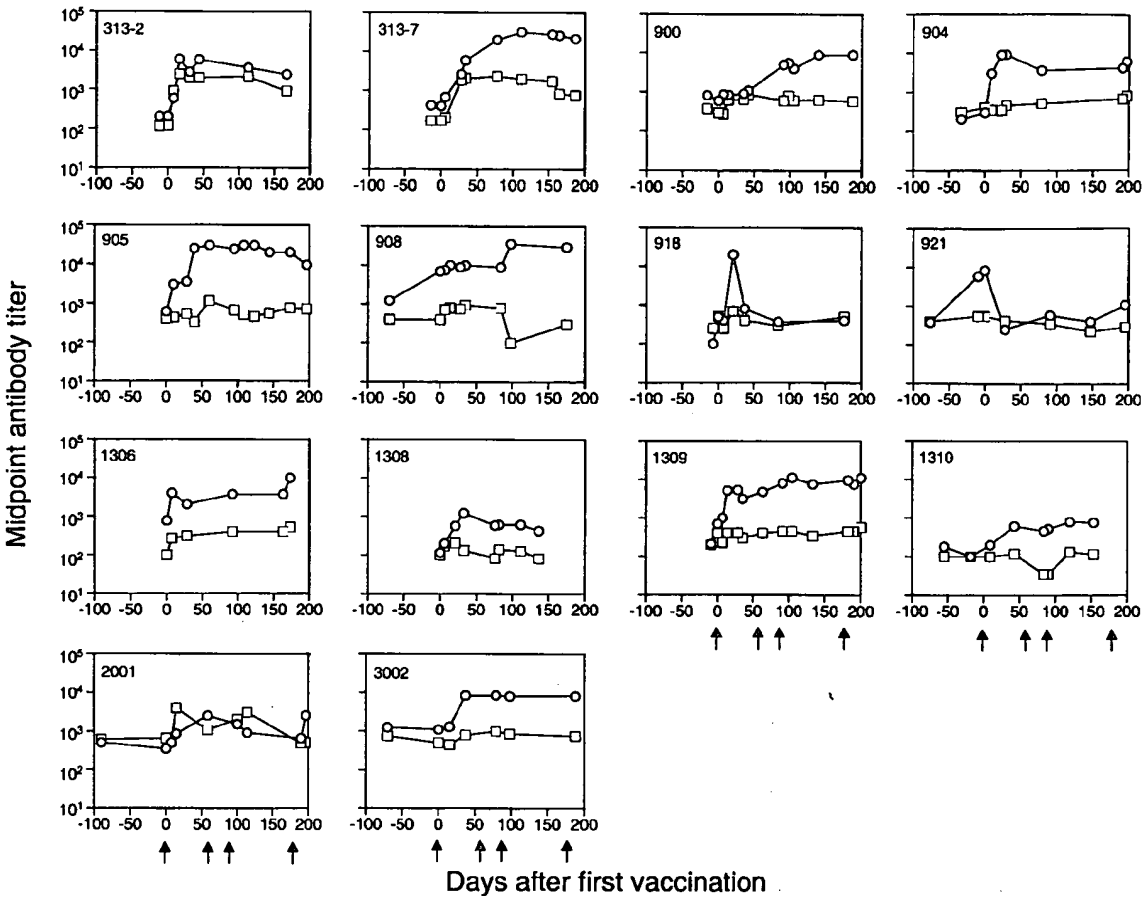


FIG. 2. Vaccination boosted binding antibody responses to HIV-1 antigens. The levels of anti-gp120 (circles) and anti-p24 (squares) antibodies in plasma were quantified using a standard ELISA and are expressed as a midpoint antibody titer.

0.03% of $CD8^+$ T cells to produce IFN-γ, whereas 5 μg/ml of the positive control superantigen, SEB, stimulated between 1 and 20% of the $CD8^+$ T cells to produce IFN-γ (data not shown). Based on these preliminary experiments, 0.05% (mean + 1 standard deviation) of IFN-γ-producing cells was determined to be significantly above the background and considered a positive value.

Persistent HIV-1-specific $CD8^+$ T-cell responses were in-

TABLE 2. Immune responses after vaccination

Subject no.	Antibodies[a]		Stimulation index[b]		Specific CD8− T cells[c]			
	Anti-p24	Anti-gp120	Anti-p24	Anti-gp160	Gag	Pol	Env	Pol/Nef
313-2	++	++	++	++	−	−	−	−
313-7	+	++	++	++	+	−	−	−
900	−	! !	! !	! !	! ! !	! !	! ! !	! !
904	−	++	++	−	++	+	−	−
905	−	++	−	+	−	+	−	−
908	−	++	++	++	+	+	−	++
918	−	! ! !	−	−	−	−	−	! !
921	−	−	−	−	+	−	+	+
1306	−	+	−	−	+	−	−	+
1308	−	++	+	+	−	−	−	−
1309	−	++	+	+	−	++	−	++
1310	−	+	−	−	++	−	−	−
2001	+	+	−	−	++	−	−	+
3002	−	+	++	+	−	−	−	−

[a] Antibody titer increase: −, <0.5 log; +, between 0.5 and 1.0 log; −+, between 1.0 and 2.0 logs; +−+, m more than 2.0 logs.
[b] T-cell proliferation stimulation index increase: −, <5; +, between 5 and 20; ++, between 20 and 100; +++, more than 100.
[c] IFN-γ^+ $CD8^-$ cell increase: −, <0.05%; +, between 0.05 and 0.10%; −+, between 0.1 and 0.5%; −+−, more than 0.5%.

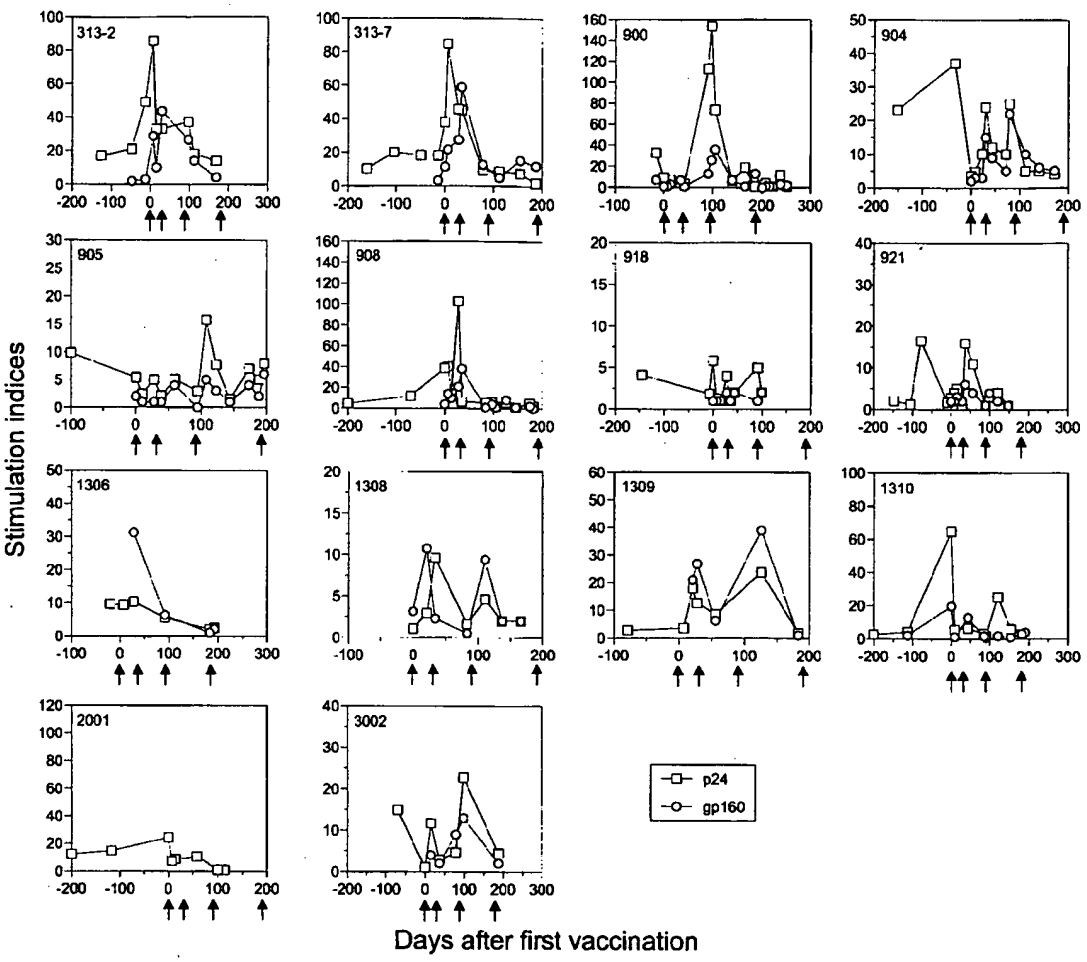


FIG. 3. Transient elevation of T helper cell responses was achieved by vaccination. T-cell proliferative responses to HIV-1 gp160 (circles) and p24 (squares) were measured using freshly isolated PBMC from each patient. The results are expressed as stimulation indexes, where control antigen stimulation was given a value of 1.

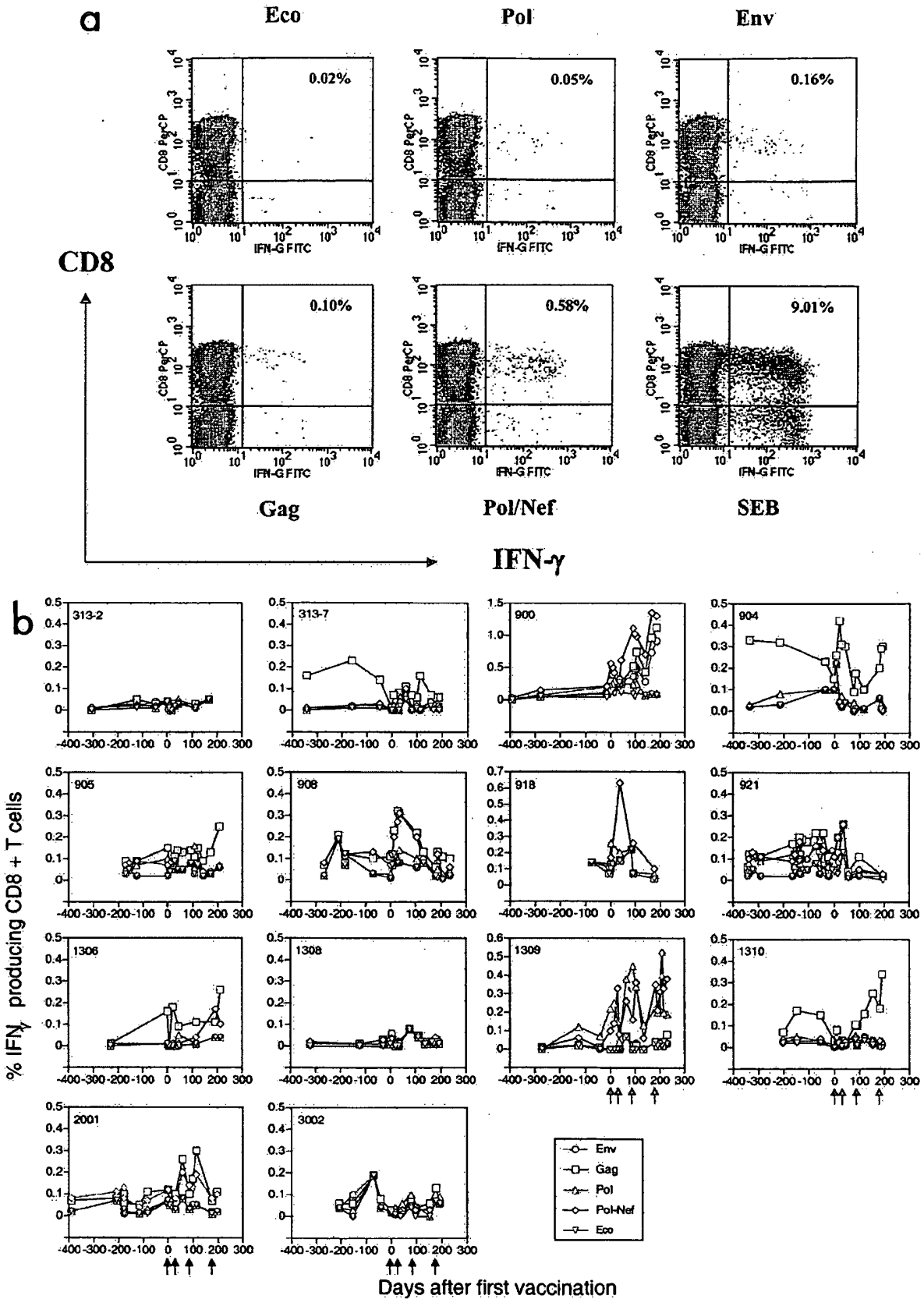
duced by vaccination in the majority of patients. The $CD8^+$ T-cell responses to vaccination were quantified longitudinally (Fig. 4b). Seven subjects (1306, 1309, 900, 904, 908, 921, and 2001) had an elevation in $CD8^+$ T-cell responses to more than one HIV-1 antigen. Four subjects (313-7, 905, 918, and 1310) had a measurable response to a single antigen, and the remaining three patients (313-2, 1308, and 3002) had no appreciable response to any antigen (Table 2). Cumulatively, 11 of 14 (78%) subjects had an increase in $CD8^+$ T-cell responses to at least one HIV-1 antigen.

Most patients who had a response recognized Gag (7 of 14) or Pol (5 of 14), and less frequently Env (2 of 14). Although more patients (8 of 14) responded to Pol/Nef, we cannot determine whether Pol or Nef was recognized. However, two patients (1310 and 918) recognized Pol/Nef but not Pol, suggesting that Nef epitopes in the ALVAC vaccine stimulated $CD8^+$ T-cell responses in at least a small number of participants. It is important to observe that these vaccine-induced HIV-1-specific $CD8^+$ T-cell responses persisted throughout the vaccine study period.

HIV-1-specific $CD8^+$ T-cell responses in these vaccine recipients were also analyzed using a major histocompatibility complex class I tetramer containing the A2/Gag and A2/Pol peptides as described (36) in five HLA-A2-positive individuals (313-2, 1306, 1308, 1309, and 3002) included in this study. In two patients (1306 and 3002), whose total number of Gag-specific $CD8^+$ T cells increased after the vaccinations, the A2/Gag tetramer-staining cells increased twofold (0.04 to 0.08%). There were similar increases in the number of A2/Pol tetramer-staining cells (0.04 to 0.09%) in another patient (1309) whose Pol-specific $CD8^+$ T cells were augmented after vaccination (data not shown).

DISCUSSION

In this therapeutic vaccination study, we evaluated the safety and immunogenicity of vCP1452 plus recombinant gp160 in 15 HIV-1-infected patients who were diagnosed and treated within 120 days after the onset of symptoms of newly acquired HIV-1 infection. Fourteen subjects completed the vaccination


a
Eco
Pol
Env
0.02%
0.05%
0.16%
CD8
0.10%
0.58%
9.01%
Gag
Pol/Nef
SEB
IFN-γ
CD8 PerCP
IFN-G FITC
b
313-2
313-7
900
904
905
908
918
921
1306
1308
1309
1310
2001
3002
% IFNγ producing CD8 + T cells
Env
Gag
Pol
Pol-Nef
Eco
Days after first vaccination

trial and have been assessed for virologic and immunologic changes pre- and postvaccination. The lack of significant (moderate or severe) adverse events establishes the safety of this vaccine regimen in this cohort of HIV-1-infected and HAART-treated patients. Importantly, these vaccines are also immunogenic. Specifically, all except one patient (93%) had increases in anti-gp120 or anti-p24 antibody titers. Nine out of 14 (64%) patients had an early and transient increase in $CD4^+$ T-cell responses to either Env or Gag, and 11 out of 14 (78%) vaccinated subjects had an augmentation of HIV-1-specific $CD8^+$ T-cell responses postvaccination.

We used a novel FACS-based intracellular cytokine staining assay to assess $CD8^+$ T-cell-specific responses. We believe that this assay represents major advantages over standard assays that were available at the time we initiated this trial. Specifically, we elected to avoid the laborious limiting-dilution assay based on chromium release (26) and the very restrictive and costly tetramer binding assay (3, 37). It is important that FACS-based intracellular cytokine staining assays using overlapping HIV-1 peptides and vaccinia virus constructs to more accurately assess immune responses have become commonplace (2). Importantly, the assay we used was highly reproducible and standardized, and the vaccinia virus vectors contained the same HIV-1 proteins as those in the vaccine being tested in this clinical trial.

Previous therapeutic vaccine studies have been done in the setting of nonsuppressive antiretroviral therapy and used monovalent vaccines. The diversity of immune responses generated by these vaccines was limited (28, 30, 34, 42, 51, 57). In contrast, the vaccine regimen that we used stimulated both humoral and cellular immunity in a majority of patients. Furthermore, $CD8^+$ T-cell responses to all four major HIV-1 gene products included in the canarypox virus vector, Env, Gag, Pol, and Nef, were boosted. It is remarkable to note that over 50% of the vaccine recipients had $CD8^+$ T-cell responses to more than one HIV-1 antigen.

Previous studies using a similar vaccine regimen, albeit an earlier generation of ALVAC vaccine, have shown this strategy to be capable of eliciting both humoral and cellular immune responses in a proportion of vaccinated seronegative persons (7, 12). The strategy of priming with ALVAC containing a single HIV *env* gene and boosting with rgp120 elicited cumulative envelope-specific CTL responses in over one third of seronegative vaccine recipients (7, 16). When a multivalent canarypox vector was used, 61% of the seronegative vaccine recipients had a CTL response detected at least one time point postvaccination (12). The cellular immune responses, however, were usually transient.

In the current study, we have demonstrated a more sustained $CD8^+$ T-cell response to multiple HIV-1 antigens in a larger proportion of vaccine recipients. Our current vaccine regimen enhanced HIV-1-specific $CD8^+$ T-cell responses in 78% of vaccine recipients. Furthermore, these $CD8^+$ T-cell responses persisted over the study period once they became detectable. Similar to the results observed in the uninfected individuals, the most dominant response tended to be specific to the Gag protein, although every antigen included in the vCP1452 elicited a $CD8^+$ T-cell response. In most vaccine recipients, the magnitude of increase in HIV-specific $CD8^+$ T cells was between 0.1 and 0.5%, which is equivalent to a frequency of 1,000 to 5,000 per 10^6 $CD8^+$ T cells. This level of antigen-specific $CD8^+$ T cells is similar to levels observed in acutely infected patients by others (2), but less than the magnitude of HIV-1-specific $CD8^+$ T cells in long-term nonprogressors (25, 43).

The size of the cohort limits our ability to dissect factors that would predict a favorable $CD8^+$ T-cell-mediated immune response to vaccination. However, all three subjects without a measurable $CD8^+$ T-cell response had at least one HLA-A2 allele. This is an unexpected finding, considering that the Pol/Nef portion of ALVAC 1452 is based on known HLA-A2-restricted epitopes. However, it has been noted recently that responses to HIV-1 in the acute setting may be quite different from the "immunodominant" epitopes identified in chronically infected individuals (2). Duration of infection pretreatment, duration of treatment, and HLA class I homozygosity at the A, B, or C locus did not predict a more or less robust $CD8^+$ T-cell-mediated immune response, either quantitative or qualitative, to vaccination. As the size of this pilot phase II study was limited, we do not believe it is possible to draw conclusions regarding positive or negative predictors of response until substantially larger cohorts are similarly studied in controlled randomized trials.

The HIV-1-specific $CD4^+$ T-cell responses, as measured by lymphocyte proliferation to Gag, were transiently elevated in 64% of the vaccine recipients. In one third of the patients who had a response, the initial response was boosted for a second time but not sustained. The preponderance and kinetics of these responses are similar to those observed in seronegative individuals receiving similar vaccines (7). It remains unclear why these responses do not persist. One explanation may be that these memory HIV-1-specific $CD4^+$ T cells are both limited in repertoire and more prone to activation-induced cell death, or apoptosis. Therefore, subsequent stimuli would be met by a limited $CD4^+$ T-cell-mediated memory response. Whether this is vaccine specific or global for all HIV-1 antigens remains unanswered.

In agreement with previous studies using a different formulation of rgp160 (28, 42, 57), we observed anti-gp120 antibody responses in all except one patient. However, few subjects demonstrated an induced antibody response to Gag (p24). These data suggest that the rgp160 component was capable of

FIG. 4. (a) Precise quantitation of HIV-specific $CD8^-$ T-cell responses using the intracellular cytokine staining assay. PBMC from one HIV-infected individual were stimulated by vaccinia virus expression negative control Eco, HIV antigens (Env, Gag, Pol, and Nef) and positive control SEB first, following by staining with anti-CD3, -CD4, -CD8 and -IFN-γ antibodies. The percentages of antigen-specific $CD8^+$ T cells were enumerated and are expressed as a percentage on the upper right quadrant of each plot. (b) Persistent increase in HIV-1-specific $CD8^+$ T cells during vaccination. Longitudinal PBMC samples from each vaccine recipient were used for assessing HIV-1 or control antigen-specific $CD8^+$ T-cell responses. The results were summarized as the percentage of IFN-γ-producing $CD8^+$ T cells to Env (circles), Gag (squares), Pol (triangles), and Pol/Nef (diamonds) after the value for background Eco (inverted diamonds) was subtracted.

eliciting a humoral response to Env and that the viral vector, ALVAC 1452, is a poor inducer of a humoral response. Alternatively, and less likely, is that ALVAC 1452 does induce an Env antibody response but no Gag antibodies due to differences in antigen expression and processing. However, the kinetics of antibody response in our subjects were similar to that seen in seronegative subjects receiving gp120 vaccination, suggesting that we are indeed seeing a response to the recombinant protein.

The binding antibody titers reached peak levels after the third vaccination and remained at plateau levels during subsequent vaccination (12, 31). Induced anti-gp120 antibody levels exhibited apparently accelerated decay in subject 918, as did prevaccination anti-gp120 levels in subject 921. Neither subject had evidence of conditions associated with accelerated loss of serum proteins (i.e., nephrosis) or rapid extravasation to the extravascular space. The underlying mechanism of this effect in these subjects remains unclear.

In the limited number of subjects studied, we were unable to demonstrate that the antibodies induced by vaccination neutralized CCR5- and CXCR4-using laboratory strains in vitro. Compared to primary isolates, these viruses are generally more neutralizable (33) and have envelopes more analogous to the exogenous immunogens in the vaccines (32). That the induced antibodies did not neutralize these strains discouraged further such studies. With further improvement in the formulation of immunogens, such as using trimeric gp140 capable of eliciting HIV-specific neutralizing antibodies in mice (60), it is possible that neutralizing antibody responses may be induced by vaccination in human subjects. Though antibody-associated cellular cytotoxicity has recently been described as a potential mechanism of virologic control during primary HIV-1 infection (18), we have not yet assessed whether the antibodies induced by vaccination with ALVAC 1452 and rgp160 have similar properties and believe these experiments are beyond the scope of the original study.

We occasionally observed discordance between vaccine-induced $CD4^+$ T-cell immune responses as measured by lymphocyte proliferation, $CD8^+$ T-cell-mediated immune responses as measured by intracellular IFN-γ staining, and humoral responses to vaccination. We hypothesize that a minority of subjects, such as 313-2, responded to vaccination with a dominant Th2 response, accounting for a robust humoral (Gag) and T-cell proliferative response (Gag and Env) without a $CD8^+$ T-cell response (8, 17, 45). On the other hand, if we assume that the Env humoral response was due to the rgp160 component of the vaccine regimen, then most of the subjects appeared to respond in a Th1-dominant pattern of various degrees, with a $CD8^+$ T-cell induced immune response, no humoral response to Gag, and a lymphocyte proliferative response, likely mediated by interleukin-2 or other Th1 cytokines (8, 17, 45).

In assessing the immunogenicity of the vaccine, it is important to determine whether the immune responses observed were due to occasional bursts of viremia, i.e., autovaccination, instead of induction by the exogenous immunogens. Three subjects had one episode of measurable plasma viremia of 50 to 100 copies/ml during the period of vaccination. A fourth patient (900) was admittedly nonadherent to HAART and appeared to have augmented already induced HIV-1-specific $CD4^+$ and $CD8^+$ T-cell responses after the third and fourth vaccinations. In this subject, the first detectable $CD8^+$ T-cell response appeared immediately after the first injections of vaccines, about 60 days prior to a detectable viremic episode. In subject 908, the initial $CD4^+$ and $CD8^+$ T-cell responses appeared to correlate with the timing of his viremic episode, but the second augmentation of $CD8^+$ T-cell responses was observed in the absence of any detectable plasma viremia. In contrast, subject 3002 never had detectable $CD8^+$ T-cell responses despite also having a viremic episode during vaccination. The isolated viremic episode in subject 1306 had little impact on the kinetics of the immune responses measured in this subject. Furthermore, viremia in these three subjects had little influence on the anti-p24 antibody or the HIV-1 specific $CD4^+$ T-cell mediated immune responses observed.

More importantly, eight patients had augmentation of HIV-1-specific $CD8^+$ T-cell responses in the absence of any detectable viremia. Thus, in most vaccinated individuals, the elevated immune responses postvaccination appear to be induced by the exogenously provided immunogens instead of autologous viruses, and the occasional viremic episodes are unlikely to have been the major determinants of immunogenicity. It is also difficult to determine whether these occasional isolated viremic episodes were directly related to immune stimulation caused by the vaccines or reflect the well-documented occurrence of low-level viremia due to incomplete suppression of viral replication during HAART.

Although we observed significant immunogenicity of the vaccine regimen, the unique features of this cohort of vaccine recipients should not be overlooked. All subjects included in the study were treated early, within 120 days of the onset of symptoms of acute HIV-1 infection. Early treatment may have preserved HIV-1-specific immunity, which allowed boosting by vaccination. Indeed, immunological manipulations, such as vaccination and structured treatment interruption, have been shown to be effective in the setting of acute lentivirus infection (22, 29, 46). It is yet to be demonstrated whether such immune-based strategies will be successful in chronic HIV-1 infection.

Although the ALVAC vCP1452 plus rgp160 regimen can induce both humoral and cellular immune responses in HIV-1-infected individuals on HAART, the limited magnitude of the $CD8^+$ T-cell response and the lack of persistence of T helper cell responses may limit the utility of this particular immunogen in this clinical setting. Nevertheless, we have demonstrated the feasibility of using multivalent vaccines as therapeutic modalities in HIV-1-infected patients receiving HAART. The promising safety and immunogenicity data generated from this preliminary study support further controlled studies in a variety of HIV-1-infected patient cohorts to better assess the efficacy of this adjunctive vaccine strategy.

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Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know

Giuseppe Pantaleo & Richard A Koup

The field of vaccinology began in ignorance of how protection was instilled in vaccine recipients. Today, a greater knowledge of immunology allows us to better understand what is being stimulated by various vaccines that leads to their protective effects: that is, their correlates of protection. Here we describe what is known about the correlates of protection for existing vaccines against a range of different viral diseases and discuss the correlates of protection against disease during natural infection with HIV-1. We will also discuss why it is important to design phase 3 clinical trials of HIV vaccines to determine the correlates of protection for each individual vaccine.

Different generations of vaccines

Vaccines that have been developed for a range of different infectious diseases and the approaches for identifying immune correlates of protection have undergone substantial changes since the inception of the field of vaccinology. First-generation vaccines were exclusively live, attenuated pathogens. Because of safety concerns, second-generation vaccines—chemically or physically inactivated pathogens—were later developed. Purified or synthetic proteins represent a third generation, and recent advances in molecular biology and genetic engineering have led to the development of the fourth vaccine generation, which includes DNA- and virus vector–based vaccines.

Until recently, experimental approaches to vaccine development have been mostly empirical. Most vaccine candidates have entered clinical trials in the context of a limited knowledge about their ability to stimulate immune responses and a poor understanding of the types of immune responses that they might elicit to confer protection. More recently, delineation of the immune correlates of protection during natural infection and after vaccination has become a fundamental step in the process of vaccine development. This has been possible thanks to a better understanding of the mechanisms underlying the generation of immune responses and the development of powerful technologies to monitor the vaccine-induced immune response.

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Vaccines and immune responses

Vaccine-induced immune responses can vary substantially depending on the nature of the immunogen. The immune response generated by live-attenuated vaccines is generally very similar to that elicited by natural infection and thus includes both antibodies able to prevent infection of target cells (neutralizing antibodies) and cell-mediated immunity. Killed virus vaccines and purified synthetic proteins preferentially elicit neutralizing antibodies and $CD4^+$ T-cell responses but not $CD8^+$ cytotoxic T lymphocytes (CTLs). Replication-defective virus-based vectors alone, and to a greater extent in combination with DNA, predominantly stimulate CTLs and $CD4^+$ T-cell responses but are less efficient at eliciting antibodies. A number of live attenuated virus, killed virus and protein-based vaccines are clearly effective against certain human viruses (Table 1). These vaccines provide protection through the generation of neutralizing antibodies, cell-mediated virus-specific immune responses or both[1–5], indicating that these responses represent the immune correlates of protection against a range of human viruses. Notably, all of the vaccines able to prevent chronicity during natural infection are associated with the generation of virus-specific neutralizing antibodies. Indeed, there is wide consensus that antibodies are crucial for preventing chronic infection, whereas cell-mediated responses can potentially control the infection in instances where chronicity is not abrogated. That antibodies may also have beneficial effects against disease progression cannot be ruled out, particularly as recent studies (discussed later) suggest a protective effect of antibodies during chronic infection.

Virus biology and vaccine protection

Knowledge of pathogen biology may aid in understanding why certain viruses are more effective at establishing chronic infection and why different viruses have different susceptibilities to the components of the immune response. Two classes of virus can be distinguished: those that are normally cleared during natural infection (acute viruses) such as smallpox, polio, measles, mumps, rubella and influenza, and those that normally establish chronic infection, such as Epstein-Barr virus (EBV), cytomegalovirus (CMV), hepatitis (B and C), herpes simplex virus (HSV) and HIV-1. Neutralizing antibodies can prevent infection with both acute and chronic viruses. Currently available vaccines are mostly effective against acute viruses, and protection is indeed conferred through neutralizing antibodies. However, there is strong evidence that cell-mediated immunity is crucial in the control of established chronic virus infections, such as CMV[6] and EBV infection[7]. Vaccine-induced cell-mediated immune responses

Table 1 Correlates of immune protection in different virus infections

Virus	Type of vaccine	Vaccine-induced protective immunity	Mechanisms of immune control during virus infection
Smallpox	Live	Antibodies, CTL	CTL
Rabies	Killed virus	Antibodies	Antibodies, CD4, CTL
Polio	Live or killed virus	Antibodies	Antibodies
Measles	Live	Antibodies; CTL	Antibodies, CD4, CTL
Mumps	Live	Antibodies	Antibodies
Rubella	Live	Antibodies	Antibodies
Varicella zoster	Live	Antibodies; CTL	Antibodies, CTL
Influenza	Protein	Antibodies	Antibodies, CD4, CTL
Hepatitis A	Killed virus	Antibodies	Antibodies, CD4, CTL
Hepatitis B	Protein	Antibodies	Antibodies, CD4, CTL
Human papillomavirus	VLP	Antibodies	CD4, CTL
Hepatitis C	–	–	CD4, CTL
Cytomegalovirus	–	–	CD4, CTL
Epstein-Barr virus	–	–	CD4, CTL
Herpes simplex virus types 1 and 2	–	–	CTL
HIV-1 and HIV-2	–	–	CD4, CTL
Human herpesvirus 6			Antibodies, T cells

have also been shown to control chronic disease in the simian immunodeficiency virus (SIV) model of AIDS[8–12].

Correlates of protection in HIV-1 infection

The susceptibility of HIV-1 to the components of the antiviral immune response is shown in Figure 1. HIV-1-specific cell-mediated immunity is generally unable to adequately control virus replication. A series of studies have helped to characterize $CD4^+$ and $CD8^+$ HIV-1-specific responses and to understand why these responses may be poorly effective. Despite the loss of HIV-1-specific helper $CD4^+$ T cells with the capacity to proliferate, HIV-1-specific $CD4^+$ T cells that secrete IFN-γ remain abundant[13]. Therefore, the problem is not a lack of HIV-1-specific $CD4^+$ T cells but rather a skewing toward one functional population of $CD4^+$ T cells. The functional heterogeneity of antiviral memory T-cell responses against different virus infections such as HIV-1, EBV and CMV[14–17] has recently been delineated. As EBV and CMV infections are effectively controlled and cell-mediated immunity seems to play the predominant role in this control, it has been assumed that the EBV- and CMV-specific T cells are prototypes of effective immune responses, whereas HIV-1-specific T cells represent ineffective immune responses. EBV- and CMV-specific $CD4^+$ T-cell responses are characterized by the presence of three functionally distinct populations: cells that secrete IL-2 but not IFN-γ, cells that secrete both IL-2 and IFN-γ, and cells that secrete IFN-γ but not IL-2 (ref. 16). These functionally distinct cell populations are associated with different conditions of antigen persistence and antigen load. The single IL-2 response is typical of antigen clearance, the single IFN-γ response is typical of antigen persistence and high antigen load, and the polyfunctional IL-2 plus IFN-γ response is typical of protracted antigen exposure and low antigen load. Notably, the HIV-1-specific $CD4^+$ T-cell responses in subjects with nonprogressive disease were polyfunctional, similar to the EBV- and CMV-specific $CD4^+$ T-cell responses [16].

Similar considerations can be made for HIV-specific $CD8^+$ T-cell responses. Lack of viral control occurs despite high frequencies of HIV-1-specific IFN-γ-secreting $CD8^+$ T cells[18] and the recognition of multiple epitopes within virus proteins[19]. Substantial qualitative differences have been found in the type of antigen-specific $CD8^+$ T cells involved in the response against HIV-1 as compared to CMV and EBV. In HIV-1 infection there is a skewing of memory $CD8^+$ T cells toward those that secrete IFN-γ, whereas there is no evidence of virus-specific $CD8^+$ T cells with the capacity to proliferate[20] or to secrete IL-2 (G.P. and A. Harari., unpublished observations). The presence of virus-specific $CD8^+$ T cells that can proliferate and secrete IL-2 seems to be associated with low levels of antigen load and virus control, as in CMV and EBV infections and in HIV-1 infection in individuals with nonprogressive disease.

Overall, chronic, progressive HIV-1 infection is associated with a monofunctional T-cell response characterized by high frequencies of virus-specific $CD4^+$ and $CD8^+$ T cells that secrete IFN-γ[15,16]. In contrast, EBV and CMV infections and nonprogressive HIV-1 infection are characterized by polyfunctional (IL-2 plus IFN-γ secreting) memory $CD4^+$ and $CD8^+$ T-cell responses reflecting the presence of memory T cells at different stages of differentiation[16]. These data suggest that the effectiveness of virus-specific immune responses is not based exclusively on the quantity but rather on the quality of $CD4^+$ and $CD8^+$ T cells.

Several observations support this hypothesis. (i) Virus-specific $CD8^+$ T-cell responses have been detected in individuals who were exposed to HIV-1 but remained uninfected[21–23]. (ii) The preservation of HIV-1-specific helper $CD4^+$ T-cell responses is associated with virus control after interruption of antiviral therapy in HIV-1-infected individuals treated early during primary infection[24]. (iii) Depletion of $CD8^+$ T cells results in a loss of virus control in monkeys infected with SIV, and SIV replication is rapidly controlled after $CD8^+$ T-cell responses are restored[25,26]. (iv) A small percentage of HIV-1 infected individuals do not experience disease progression in the absence of treatment—the so-called long-term nonprogressors (LTNPs). (v) HIV-1-specific $CD4^+$ and $CD8^+$ T-cell responses are preserved in LTNPs and, more importantly, the memory T-cell responses are qualitatively very similar (polyfunctional) to the EBV- and CMV-specific immune responses[16]. Therefore, under certain conditions HIV-1-specific cell-mediated immunity may effectively control virus replication during established chronic infection.

With regard to the role of humoral immunity in HIV-1 infection, the demonstration that passive immunization with neutralizing antibody can prevent establishment of chronic infection in chimpanzees acutely inoculated with HIV-1 dates back to 1992 (refs. 27,28). It is still unclear, however, whether neutralizing antibodies have a substantial role in the control of chronic, established HIV-1 infection, although there is some evidence from the SIV model that neutralizing antibodies may influence chronic steady-state viremia[31]. The limited effect may be related to the very rapid escape from neutralizing antibodies that occurs in infected individuals[29,30].

However, the correlates of protection involved in preventing disease progression during natural infection with HIV-1 do not necessarily reflect those that would be protective in the presence of pre-existing vaccine-induced immunity. In the absence of clinical trials, it is difficult to predict exactly how pre-existing immunity would affect the outcome of HIV-1 infection.

HIV-1 vaccine candidates

Most of the first candidate HIV-1 vaccines were based entirely or partially on the use of recombinant envelope proteins, with the intent of stimulating neutralizing antibodies. Despite the discovery that these candidates were unable to elicit antibodies that could neutralize primary isolates of HIV-1 (the proposed immune mechanism of protection) during phase 1 and 2 testing, one candidate vaccine was pushed into phase 3 efficacy testing. Not surprisingly, this vaccine did not show efficacy in large-scale clinical trials[32]. Over the last ten years, it has become clear that HIV-1 evades neutralizing antibodies through a variety of mechanisms[29,30,33,34], and there is active research aimed at discovering ways to overcome the apparent difficulty in stimulating a broad neutralizing antibody response to HIV-1.

Most vaccines currently in the development pipeline are designed to stimulate strong cell-mediated immune responses to HIV-1[36–39]. These include several different viral vectors with or without a DNA prime or a recombinant antigen boost[40–42]. Because several of the candidate vaccines have already, in phase 1 and 2 testing, shown an ability to stimulate an immune response that is a reasonable correlate of protection, there is a rational justification for proceeding with some of these candidates to phase 3 efficacy and correlates trials[44–48].

It is likely that not every vaccine that stimulates HIV-1-specific T-cell immunity will prove equally efficacious or even have the same correlate of protection. Although stimulating HIV-1-specific $CD4^+$ T-cell responses with a vaccine would seem important (these responses would help with both B-cell and $CD8^+$ T-cell responses), the fact that HIV-1-specific $CD4^+$ T cells are preferentially infected during natural HIV-1 infection suggests that their existence at the time of initial infection with HIV could be detrimental by providing the optimal substrate for HIV-1 replication[49]. It will therefore be important to carefully dissect the positive and negative correlates of immune protection in future trials of T cell–stimulating vaccines.

Immunologic monitoring

With regard to the humoral immune response, the fundamental requisite of a vaccine that is capable of 'sterilizing immunity' (prevention of infection) is that it elicits both high titers of neutralizing antibodies, and antibodies with broad neutralizing activity.

For cell-mediated immunity, the situation is more complex. The observations supporting the protective role of cell-mediated immunity against disease progression remain predominantly indirect[21–26]. There is still no direct experimental evidence (as has been provided, in the case of neutralizing antibodies, by passive immunization studies[27]) that HIV-1-specific cellular immunity prevents disease progression. However, if we accept the assumption that the EBV- and CMV-specific and the HIV-1-specific immune responses observed in subjects with nonprogressive disease are protective, a vaccine should stimulate polyfunctional (IL-2 plus IFN-γ) $CD4^+$ and $CD8^+$ T-cell responses to be effective against disease progression (Pantaleo, G. *et al*, unpublished observations (G.P., S. Zimmerli and A. Harari)[16].

Recent advances in delineating the functional complexity of both $CD4^+$ and $CD8^+$ T cells should prompt a re-evaluation of strategies currently used to monitor vaccine-specific immune responses. Although measuring IFN-γ-secreting cells after antigen-specific stimulation using ELISpot assays can provide information on the immunogenicity of a vaccine, it may be insufficient for defining an immune correlate—determining the functional diversity of the vaccine-induced immune response may be necessary. Furthermore, the detection of IFN-γ-secreting cells may provide limited information on the durability of the vaccine-stimulated immune response. In fact, most current vaccine strategies should have a limited duration of vaccine antigen expression. Secretion of IFN-γ is typical of the early (effector) phase of immune response generation but will decrease with antigen clearance. In contrast, IL-2 secretion is typical of the long-term memory (predominantly $CD4^+$) T-cell response and thus it may be an important cytokine signature in long-term memory T cells[50]. Finally, we cannot exclude the possibility that certain vaccines will be poor inducers of IFN-γ responses while stimulating dominant IL-2 responses. Therefore, the assessment of vaccine-specific responses must be extended to detect IL-2-secreting cells. Advances in the development of multiparameter flow cytometry now allow for simultaneous assessment of multiple cytokines produced from antigen-specific $CD4^+$ and $CD8^+$ T cells. Thus, although ELISpot assays may be suitable for initial screening of the immunogenicity of large numbers of samples from vaccine trials, multiparameter flow cytometry may be needed to fully characterize vaccine-induced memory T-cell responses.

Design of phase 3 vaccine trials

Initial phase 3 trials of new vaccine products can be designed to determine efficacy or a combination of efficacy and the correlate of immune protection. Obviously, there is no correlate in the absence of demonstrated efficacy, but inherent in these two trial designs is the fact that efficacy/correlates trials may require more subjects than straight efficacy trials. How many more depends, to some extent, on the ability of the vaccine to stimulate a measurable immune response. It will take many more patients to determine a correlate of protection if an immune response can only be measured in 30% of vaccine recip-

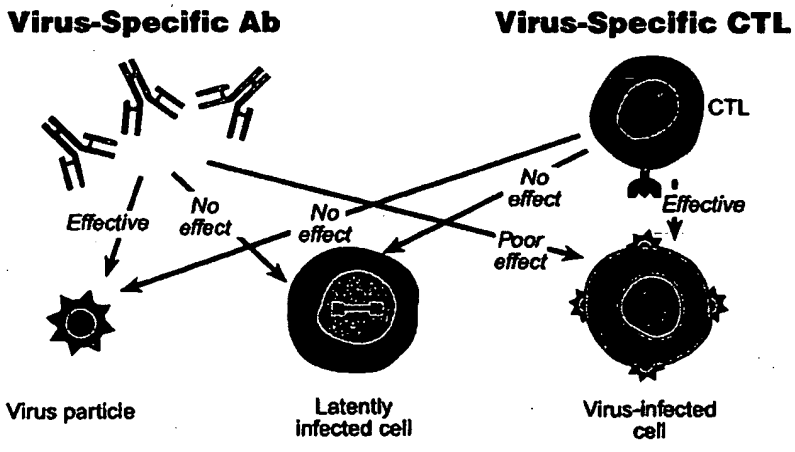

Figure 1 Schematic representation of the effectiveness of the components of the antiviral immune response against different forms of HIV-1. Neutralizing antibodies are efficient in blocking virus particles but poorly effective against cell-associated virus, such as virus-infected cells. Some CTLs are effective against virus-infected cells but not against free virus particles. Neither antibodies nor CTLs are effective against latently infected cells.

BOX 1 – CORRELATES OF IMMUNE PROTECTION

What we know

- Adaptive immune responses, in the form of neutralizing antibodies and virus-specific $CD4^+$ and $CD8^+$ T cells, are the principal correlates of immune protection for most infections and vaccines.
- Neutralizing antibodies are the immune correlates of protection from infection.
- Cell-mediated immune responses are the immune correlates of protection from disease.
- Studies of passive immunization and immune depletion in animal models strongly suggest that both neutralizing antibodies and cell-mediated immune responses may provide effective protection from infection and from disease progression in HIV-1 infection.
- The effectiveness of antiviral memory T cells is dependent upon both the quality and the number of antigen-specific T cells.
- Polyfunctional (IL-2 plus IFN-γ) $CD4^+$ and $CD8^+$ T-cell responses, and not monofunctional IFN-γ responses, are associated with at least partial virus control.
- Immunologic monitoring of vaccine-induced T-cell immune responses cannot be limited to cells that secrete IFN-γ and should be extended to include cells that secrete IL-2.

What we don't know

- How to induce high titers of neutralizing antibodies.
- Whether any of the vaccines being currently developed will elicit cellular immune responses that will correlate with protection from infection or disease progression.

What we should know

- The type (poly- or monofunctional) of $CD4^+$ and $CD8^+$ T-cell responses elicited by the vaccines currently being developed.
- The breadth of the vaccine-induced $CD4^+$ and $CD8^+$ T-cell responses.
- The best combination of vaccines that in the prime-boost immunization strategies will stimulate an immune response similar to that thought to confer protection from disease progression.

ients than if it can be measured in 90% of recipients. Therefore, a combination of a potent vaccine and a sensitive and specific assay to measure even low levels of a vaccine-induced immune response will combine to lessen the number of subjects needed to prove an immune correlate of protection, potentially avoiding any need to add subjects to the efficacy trial.

There are compelling reasons to design initial phase 3 trials to determine the correlates of protection, some theoretical and others practical in nature. For one thing, despite the potential increased size and complexity of a correlates trial, including the power to determine a correlate does not result in any loss in the ability to determine efficacy. A disappointing result would be that a vaccine could be proven to be efficacious while the correlate was unknown. This is no different from the situation that existed during the early days of vaccinology. A more academic consideration is that determining the correlate of immune protection provides insight into the mechanism of protection, although it certainly does not prove cause and effect. Inherent in the fact that an immune response only 'correlates' with protection is the fact that the measured immune response may be a surrogate for some other (unmeasured) immune response (or genetic factor) that provides the true mechanism for protection. This said, the initial identification of a correlate can lead to further investigation that could ultimately determine the mechanism and lead to further rational vaccine design.

The most practical reason for determining a correlate of protection, however, is that it allows future trials to proceed more readily. If a strong correlate is found, then future trials of the vaccine can use that correlate as the endpoint, streamlining the testing process

Final considerations

Current knowledge about the correlates of protection is summarized in Box 1. Whether or not there are sufficient scientific data to support the testing of the current vaccines in large phase 2 or phase 3 efficacy trials has recently been the subject of extensive and sometimes harsh debate. The lack of understanding of some crucial scientific questions (such as how to generate neutralizing antibodies), the fact that current HIV vaccine candidates may not protect from infection, and the absence of definitive experimental evidence that certain types of immune response are indeed immune correlates of protection all favor the view that more basic research is needed before current vaccine candidates can be moved into large efficacy trials. However, it is also unclear what data from which animal model of HIV-1 infection are most relevant to human infection and vaccine protection. Notwithstanding the importance of supporting and reinforcing basic science studies to inform the direction of HIV vaccine development, it would be inappropriate to conclude that no current vaccine strategies will provide any data of importance to future vaccine development. Therefore, large phase 2 or phase 3 clinical trails designed to address the issues discussed above are crucial to hasten the future development of HIV vaccines. Identification of the correlates of protection within the vaccine trials will certainly depend upon the quality of the candidate vaccines. For example, weak vaccines are unlikely to provide protection, and as such will not generate any immune correlate. In contrast, cellular responses stimulated by potent vaccines are more likely to provide protection and a measurable correlate. Therefore, a detailed qualitative and functional characterization, rather than the simple detection of the vaccine-specific immune response, will be required to determine the immune correlates of protection against HIV.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Viremia control following antiretroviral treatment and therapeutic immunization during primary SIV_{251} infection of macaques

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Prolonged antiretroviral therapy (ART) is not likely to eradicate human immunodeficiency virus type I (HIV-I) infection. Here we explore the effect of therapeutic immunization in the context of ART during primary infection using the simian immunodeficiency virus (SIV_{251}) macaque model. Vaccination of rhesus macaques with the highly attenuated poxvirus-based NYVAC-SIV vaccine expressing structural genes elicited vigorous virus-specific CD4⁺ and CD8⁺ T cell responses in macaques that responded effectively to ART. Following discontinuation of a six-month ART regimen, viral rebound occurred in most animals, but was transient in six of eight vaccinated animals. Viral rebound was also transient in four of seven mock-vaccinated control animals. These data establish the importance of antiretroviral treatment during primary infection and demonstrate that virus-specific immune responses in the infected host can be expanded by therapeutic immunization.

Introduction

The introduction of highly active antiretroviral therapy (HAART), a combination of reverse transcriptase and protease inhibitors, has decreased morbidity and mortality in patients infected with HIV-I (ref. 1). Complex dosing schedules and undesirable side effects, however, frequently result in incomplete adherence and possibly emergence of drug-resistant viral strains.

HIV-I eradication by prolonged HAART treatment appears to be unlikely because of the persistence of a cellular reservoir of infectious HIV-I (refs 2,3). Discontinuation of HAART, in fact, results in viral rebound in most individuals chronically infected with HIV-I (ref. 4). In some patients, intermittent HAART use resulted in temporary containment of HIV-I replication[5,6]. In one of these studies , virus suppression correlated with the strength and breadth of virus-specific CD8⁺ T cell response, affirming the importance of cytotoxic response in the control of HIV-I replication[7-11]. Most of the patients with contained viremia were treated during primary infection, suggesting that early HAART treatment may limit immunological damage, as demonstrated by the preservation of CD8⁺ T cells and the CD4⁺ t cell response[12-14].

The fact that vigorous host immune response correlates with containment of HIV-I replication and the recent appreciation that prolonged HAART treatment results in a progressive decline in virus-specific response[15-17] provide the rationale to use immune-based strategies to further strengthen the host response to the virus. Here we designed a study in the SIV_{251} rhesus macaque model to assess whether early ART intervention in primary SIV_{251} infection per se results in restoration of virus-specific immune responses sufficient to contain viremia following ART discontinuation; whether vaccination of macaques enhances SIV-specific CD4⁺ T-helper and CD8⁺ t cell cytotoxic responses in ART-treated and untreated macaques; and whether the immune responses induced by vaccination correlated with the ability of the host to control viremia following ART suspension.

As a vaccine modality, we have chosen, among the several poxvirus-based vaccine candidates available[18-21], the highly attenuated poxvirus NYVAC-SIV-*gag-pol-env* (NYVAC-SIV-*gpe*) live recombinant vaccine candidate because of its ability to induce both CD4⁺ and CD8⁺ t cell responses in rhesus macaques and because of its demonstrated effectiveness as a preventive vaccine candidate[18,22].

Results

We inoculated 24 macaques intravenously with ten infectious doses of highly pathogenic SIV_{251} (R. Pal *et al.*, manuscript in preparation). The animals were divided into three groups of eight animals each (A, B and C). Animals in groups A and B, but not C, received ART. Animals in groups B and C also received three intramuscular immunizations with NYVAC-SIV-*gpe* (10^8

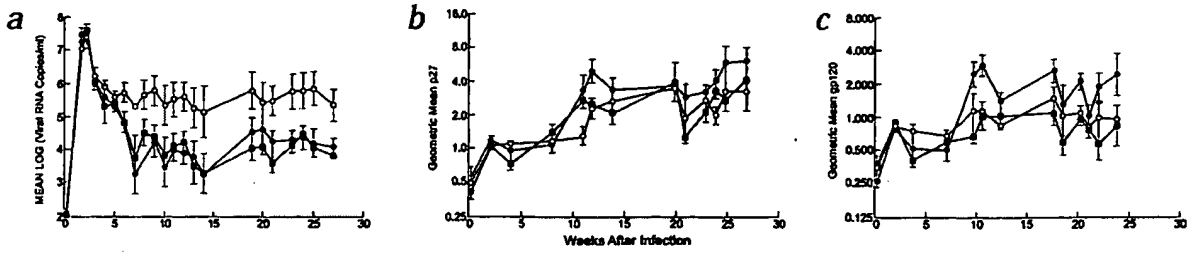

Fig. 1 Statistical analysis of the plasma virus load and proliferative response to p27 Gag and gp120 in the immunized ART-treated macaques from groups A(■), B(●) and C(○). *a*, The mean of the log plasma viremia for all animals in each group is graphically presented up to week 27. The Wilcoxon rank sum test was applied to the mean of the log of plasma viral RNA. The differences between groups A and C were significant at P = 0.02 and between groups B and C at P = 0.028 within the first 8 weeks of treatment and remained significant thereafter. Graphical representation of the geometric mean value for p27 Gag *b*, and gp120 *c*, for all animals in each group up to week 27. Differences among groups were evaluated using repeated measures ANOVA of the log of the SI over multiple times. The levels of statistical significance of these immune responses after each immunization are summarized in Table 1.

plaque forming unit each), whereas animals in group A received the mock vaccine (NYVAC) at the same time and doses. Each group included 3 Mamu-A*01 (*Macaca mulata*; nomenclature for rhesus MHC; refs. 23,24) animals. Most Mamu-A*01 animals infected by SIV recognize the immunodominant Gag peptide p11C, C→M (hereafter referred to as peptide 181; refs. 25,26) within the SIV Gag protein[27], allowing for the quantitative analysis of CD8⁺ response with a tetramer formed by 4 Mamu-A*01 molecules and peptide 181. By 2 weeks after infection, all animals developed acute viremia with peak viral loads ranging between 10^7 and 10^9 viral RNA copies per milliliter of plasma (Fig. 1*a*). All 24 macaques seroconverted to SIV_{251} Gag, Pol and Env antigens by week 8 (data not shown), but failed to develop lymphoproliferative response (LPR) to SIV_{251} p27 Gag or Env proteins during the first 10 weeks postinfection (Fig. 1*b*, *c*).

Gag-specific CD8⁺ t cell responses developed in all Mamu-A*01 animals as measured by the Mamu-A*01-p11C, C→M tetramer (Gag 181 tetramer), peaking at weeks 2 and 4 postinfection (range 0.55–3.1% of total CD8⁺ T cells in blood), consistent with previous observations[28]. These CD8⁺ T cells could be expanded following *in vitro* stimulation with the Gag 181 peptide for 7 days and the peak values of cells binding Gag-181 ranged between 4.5 and 67% of total CD8⁺ cells. The specificity of tetramer staining was demonstrated in parallel experiments using fresh and cultured peripheral blood mononuclear cells (PBMC) from Mamu-A*01-positive naive animals and SIV_{251}-infected non-Mamu-A*01 animals (data not shown).

On day 15 after SIV_{251} infection, animals in groups A and B were started on ART. Within the 25 weeks of treatment, ART reduced plasma viremia in animals from both groups A and B compared with untreated group C (Fig. 1*a*). As expected, no significant difference in viremia was observed between groups A and B (Fig. 1*a*).

At 10, 19 and 23 weeks after infection, all macaques in groups B (ART-treated) and C (untreated) were immunized intramuscularly with 10^8 pfu of NYVAC-SIV-*gpe* vaccine, whereas animals in group A (ART-treated) received 10^8 pfu of mock-NYVAC parental virus.

Immunizations with NYVAC-SIV-*gpe* increased LPR to both p27 Gag and gp120 Env antigens in macaques from group B, but not in the viremic animals from group C (Fig. 1*b*, *c* and Table 1).

As expected, mock-vaccinated, ART-treated macaques from group A had lower LPR to both p27 Gag and gp120 than the animals vaccinated with NYVAC-SIV-*gpe* in group B, and the differences between the two groups were significant after the first and second immunizations (Table 1). The low level of LPR observed in animals from group C suggest that the ability of the NYVAC-SIV-*gpe* vaccine to induce measurable CD4⁺ T-helper response was dependent on viremia suppression by ART treatment. To confirm this, we calculated the correlation coefficient from the values of LPR and virus load after each immunization. LPR to both viral antigens were detectable at levels of viremia below 10^5 copies/ml (Fig. 2).

As an indicator for CD8⁺ t cell responses induced by NYVAC-SIV, we measured Gag 181-tetramer-staining cells following NYVAC-SIV-*gpe* vaccination or mock-NYVAC vaccination in fresh and cultured PBMC of all Mamu-A*01 animals. Following the second and third immunizations with NYVAC-SIV-*gpe* vaccine (7 measurements during weeks 19–27), an expansion of CD3⁺ CD8⁺ T cells staining with Gag 181-tetramer was observed in the PBMC of animals in group B treated with ART, but not in the animals of group C, or in the mock-NYVAC-vaccinated animals from group A (Fig. 3*a*).

The kinetics of induction of virus-specific fresh or cultured Gag 181-tetramer-positive CD8⁺ T cells for each Mamu-A*01 animal is shown (Fig. 3*b*, *c*). To assess whether the Gag 181 CD8⁺ T cells had *ex vivo* effector cytotoxic T-lymphocyte (CTL) function, we measured bulk cytolytic activity in the PBMC obtained at week 23 from Mamu-A*01 animals using autologous transformed B cells pulsed with the Gag 181 peptide as a target.

Table 1 Statistical analysis of LPR to p27 Gag and gp120 following the three immunizations

Animal group	1st immunization (weeks 11, 12, 14)*	2nd immunization (weeks 20, 21, 23)*	3rd immunization (weeks 24, 25, 27)*	Overall period, weeks 11–27*
B versus C				
p27	$P = 0.021$	$P = 0.28$	$P = 0.024$	$P = 0.026$
gp120	$P = 0.012$	$P = 0.029$	$P = 0.21$	$P = 0.004$
B versus A				
p27	$P = 0.039$	$P = 0.039$	$P = 0.086$	$P = 0.0097$
gp120	$P = 0.014$	$P = 0.0095$	$P = 0.18$	$P = 0.0002$

*Time points of statistical analysis.

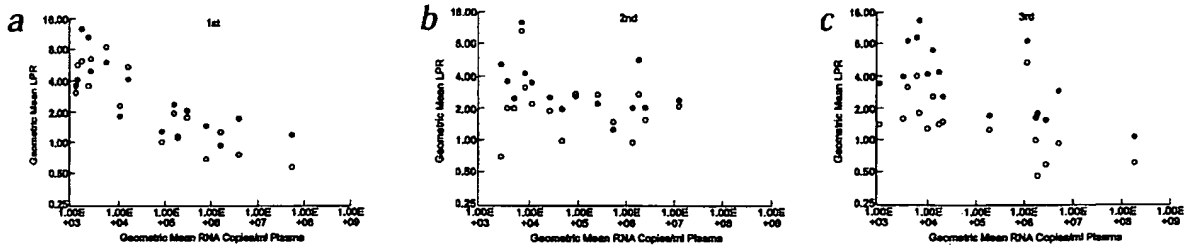

Fig. 2 Inverse correlation between LPR and viremia. In this analysis, only animals from groups B and C were included because they all received NYVAC-SIV-*gpe* vaccine. Each symbol corresponds to the geometric mean of LPR to p27 Gag (●) or gp120 (○) on the *y* axis and to the geometric mean of virus load (*x* axis) at the first and second weeks following each immunization. The Spearman rank correlation coefficients, adjusted for the mean by group, were as follows: *a*, first immunization, p27 = –0.68 (P = 0.0038), gp120 = –0.75 (P = 0.0008); *b*, second immunization, p27 = –0.42 (P = 0.12), gp120 = +0.01 (P = 0.96); *c*, third immunization, p27 = –0.51 (P = 0.041), gp120 = –0.56 (P = 0.025).

Significant CTL activity was observed in nonstimulated PBMC populations of animals 636 and 644 in group B, but in none of the animals from groups A and C (Fig. 3*b*, right). To further confirm the specificity of the Gag 181-staining CD8⁺ T cells, the PBMC from the Mamu-A*01 animals collected at the time points indicated (Fig. 3*b*) were stimulated *in vitro* with Gag 181 peptide and their ability to bind to the Mamu-A*01 Gag 181 tetramer assessed. CD3⁺ CD8⁺ T cells binding Gag 181-tetramer were expanded *in vitro* at most time points and the extent of this expansion was significantly greater in samples from animals in group B than in group C following NYVAC-SIV-*gpe* vaccination (P = 0.0045; Fig. 3*c*, left). Thus, vaccination with NYVAC-SIV-*gpe* increases the size of the virus-specific CD8⁺ T cells and the CD4⁺ helper t cell responses only in animals in which viremia is suppressed by ART.

At week 27, ART was discontinued in all animals from groups A and B. In animals of group A, plasma viral rebound occurred in all 7 remaining animals (1 animal, 637, was euthanized at week 25 because of a diabetic coma, presumably due to Didanosine (DDI) toxicity) and peaked between 10^4 and 10^6 viral RNA copies/ml within the first 3 weeks of ART suspension. Among the animals in group A, however, animals 641, 642 and 652 maintained high plasma viral load thereafter (Fig. 4*a*, top), whereas the remaining 4 animals in group A suppressed viral replication and maintained viremia below the threshold of detection of our assay (5×10^3 viral RNA copies per ml of plasma).

Of the eight animals in group B, two (647 and 655) failed to respond to ART, experienced a further increase of viremia following ART discontinuation and maintained high virus load (Fig. 4*b*, top). Of the remaining 6 animals, 5 experienced plasma re-

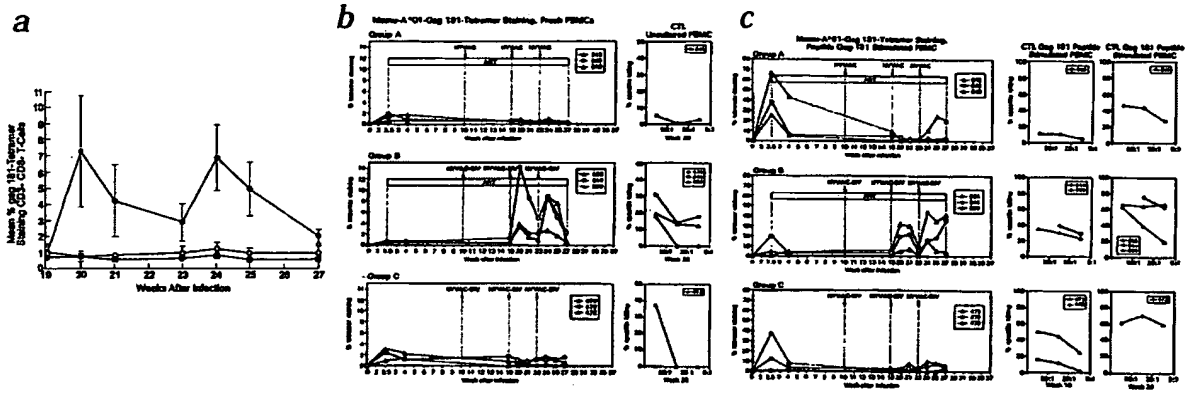

Fig. 3 Induction of CD8⁺ t cell response by NYVAC-SIV-*gpe* immunization. *a*, Graphical representation of the expansion of *ex vivo* virus-specific CD8⁺ Gag 181 t cells after the second and third immunizations. The data were obtained from all the Mamu-A*01 animals included in each group. The means of the percentages are plotted and the repeated measures ANOVA of the arcsine-transformed percentages revealed highly significant differences in the number of virus-specific CD8⁺ T cells induced by vaccination in groups B(●) versus C (○)(P = 0.00005). No significant difference was observed between groups A(■), and B(●), and C(○). *b*, Left, the percentage of Mamu-A*01-p11C, C→M-tetramer-staining cells in freshly prepared PBMC from Mamu-A*01-positive animals in groups A, B, and C (top to bottom). Data obtained during the first 4 weeks postinfection are presented as percentages of tetramer-staining cells of total CD8⁺αβ lymphocytes. Data obtained after second and third immunizations are expressed as percentages of tetramer-staining cells of total CD3⁺ CD8⁺ lymphocyte population. Panels on right represent results of bulk-killing CTL assay at the time of third immunization (week 23). [Fresh PBMC were kept overnight in presence of IL-2 and then incubated with radiolabeled autologous transformed B-cells pulsed overnight with Gag 181 peptide.] The numbers on the *x axis* represent the effector-to-target cell ratio. The killing of B cells in the absence of peptide or in the presence of an unrelated peptide ranged between 5 and 15%. The background killing is subtracted from the values of specific killing. *c*, Left, the percentage of Mamu-A*01-Gag 181-tetramer-staining PBMC following *in vitro* culture for 7 days with Gag 181 peptide. Week 0 to 4, percentage of tetramer-staining cells in CD8⁺ cell population; weeks 19 to 27, percentage of tetramer-staining cells in CD3⁺ CD8⁺ t cell population. Right, bulk-killing CTL assay using the Gag 181-peptide-stimulated PBMC obtained at the time (week 19) and 1 week after the second immunization (week 20). Statistical analysis of the data points at weeks 19 through 27, using the repeated measures ANOVA, indicated significant differences between groups B and C (P = 0.0045).

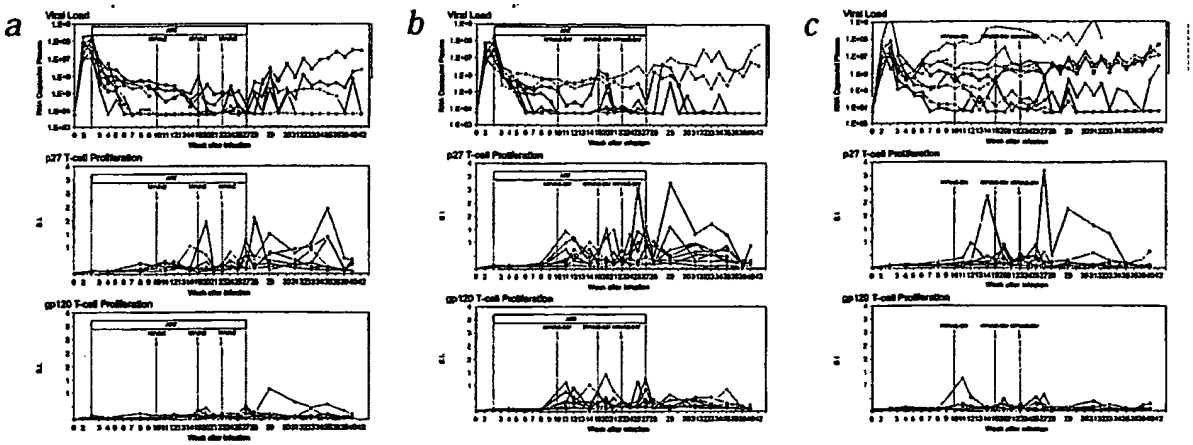

Fig. 4 Viral load, p27 Gag and gp120 LPR in all animal groups before and during immunization and ART and after ART suspension. Top panels of *a–c*, depict the viral RNA copies/ml of plasma. The time of SIV_{251} inoculation corresponds to time 0. The arrow indicates the time of each immunization. Middle and lower panels indicate the value of LPR to p27 Gag and gp120, respectively, measured at the time indicated. The numbers on the y axis correspond to SIs. a:637 (△), 640 (○), 641 (□), 642 (◇), 645 (▲), 648 (●), 649 (■), 652(◆). b: 635 (△), 636 (○), 639 (□), 644 (◇), 647 (▲), 650 (●), 655(■), 656 (◆). c:473 (△), 478 (○), 479 (□), 480 (◇), 638(▲), 643 (●), 646 (■), 653 (◆).

bound above the threshold in the first weeks and rapidly contained viremia thereafter, whereas viral rebound was not observed in animal 636 (Fig. 4*b*, top).

The overall CD4⁺ helper response to p27 Gag and gp120 before and after ART suspension is shown (Fig. 4*a–c*). Vigorous p27 Gag LPRs were measured in the four animals in group A and in the six animals from group B that contained viremia (Fig. 4*a*, *b*, middle). CD4⁺ helper responses to gp120 Env were detected only sporadically in some animals (Fig. 4*a*, *b*, bottom) after ART suspension. The level of LPR to p27 Gag was significantly higher in macaques that suppressed viremia (4 from group A and 6 from group B), than in those that did not (3 in group A and 2 in group B) before (weeks 10–27; $P = 0.00004$) and after (weeks 28–44; $P = 0.0029$) ART discontinuation, suggesting a possible correlation between Gag-specific proliferation and viremia containment, as also observed in studies of individuals infected with HIV-1 (refs. 14,29,30). This notion was further supported by the finding that animals 480 and 646 from group C, which restricted viral replication early in infection in the abscence of ART, also developed LPR to p27 Gag, and one of them developed LPR to gp120 during the time of treatment (Fig. 4*c*).

The kinetics of expansion of the Gag 181-tetramer CD8⁺ T cells following ART suspension was studied in all Mamu-A*01 animals. The largest expansion, up to tenfold, of the Gag 181-tetramer-staining CD3⁺ CD8⁺ t cell population was observed in the blood of animal 649, which restricted viral replication when ART was discontinued. This large expansion was not observed in animals 636, 644 and 650 from group B, which also restricted viral replication (Fig. 5*a*, *c*). Therefore, the contribution of this monospecific response to the containment of viral replication is dubious because consistent patterns of expansion were not found in animals that had undetectable viremia and animals that maintained high virus load (Fig. 5*a*, *c*).

Of the four animals in group A and the six animals in group B that restricted viral replication, all maintained undetectable levels of plasma-virus load within eight months from ART suspension and had no significant laboratory or clinical sign of disease progression. Among the remaining animals in group A, 641 succumbed to disease 10 months after infection, whereas animals 642 and 652 experienced a progressive loss of CD4⁺ cells. Of the remaining animals from group B, animals 647 and 655, which responded poorly to therapy, also experienced a progressive loss of CD4⁺ T cells. Of the animals in group C, four (473, 479, 638 and 643) died, indicating that NYVAC-SIV-*gpe* vaccination in the

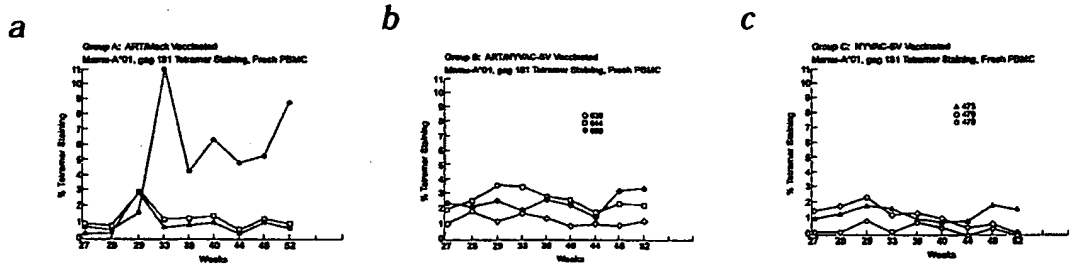

Fig. 5 Mamu-A*01-Gag 181-tetramer staining in *ex vivo* PBMC of macaques following ART discontinuation. The percentage of CD3⁺ CD8⁺ T cells binding the Mamu-A*01-Gag 181 tetramer is presented on the *y* axis versus the time following ART suspension in the 3 Mamu-A*01 animals from each group (*x* axis). *a*, Group A, animal 642 (□) did not control viremia following ART suspension, whereas animals 645 (▲) and 649 (●) did (see Fig. 4). *b*, Group B, all these animals restricted viral replication (see Fig. 4). *c*, Group C, these animals were not treated with ART.

absence of ART was of no obvious clinical benefit to these SIV_{251}-infected macaques. In conclusion, ART treatment during primary SIV_{251} infection has modified the natural course of disease in animals from groups A and B.

Discussion

Our data provide the first evidence that a highly attenuated, live recombinant poxvirus vector vaccine is able to induce and/or boost both $CD4^+$ T-helper and $CD8^+$ CTL immune responses in the context of a pharmacologically controlled lentiviral infection. This observation is in part unexpected, because it is commonly believed that infection with a given pathogen *per se* may provide the most effective response. SIV, and by extension HIV, infection differ in this regard, as both pathogens infect cells of the immune system and have developed effective strategies to interfere with the host immune response[31–33]. We have demonstrated that the induction of significant $CD4^+$ T-helper and $CD8^+$ t cell responses by the NYVAC-SIV-*gpe* vaccination were dependent on the level of viral replication. It is possible that in animals with high plasma viremia, the pool of virus-specific memory $CD4^+$ T cells is either decreased, exhausted[14] or already activated in vivo[14]. In fact, a low number of $CD4^+$ T lymphocytes responding to short-term *in vitro* stimulation with p27 Gag, as measured by intracellular expression of TNF-α, was detected in the blood of some animals with high virus load, which scored negative in lymphoproliferative assay (data not shown). A similar finding has also been recently demonstrated in humans infected with HIV (ref. 34). An alternative interpretation may be that PBMC of viremic animals harbor virus *in vitro* that could interfere with the measurement of proliferative responses. We do not favor this interpretation, however, because of the short-term nature of our assay and because in the same animals NYVAC-SIV-*gpe* vaccination also failed to expand the Gag-specific $CD8^+$ T cells, which are not SIV_{251} targets. Moreover, a discrepancy was also observed in the ability of $CD8^+$ T cells from animals in group C to proliferate *in vitro*. In fact, although a relatively high percentage of circulating Gag-specific $CD3^+$ $CD8^+$ T cells was found in the blood of these animals, these cells could not be efficiently expanded *in vitro* (Fig. 3*b*, *c*), suggesting their limited potential for proliferation.

We have demonstrated that the live, attenuated NYVAC-SIV-*gpe* poxvirus vaccine candidate represents a potent inducer/booster of $CD3^+$ $CD8^+$ T cells, as we detected up to 14% Mamu-A*01-Gag 181-tetramer-staining $CD8^+$ T cells in the blood of vaccinated animals. We used the Gag 181-tetramer-staining assay as a tool to monitor the $CD8^+$-specific immune response to the NYVAC-SIV-*gpe* vaccine. The lack of an obvious correlation with the induction of this response and the ability of the animals to control viremia following therapy suspension suggests that this monospecific Gag-epitope response may not be protective[29,35]. A broader response, which may include other dominant as well as subdominant epitopes in Gag, Pol, Env, Vif, Rev and Tat[25,36] not measured in this study, may be a better correlate of protection, as demonstrated in HIV-I-infected individuals following ART discontinuation[6]. Thus, the availability of other Mamu-A*01-restricted epitopes, as well as other genetically characterized macaques and the knowledge of the epitopes recognized by their immune systems, might further assist in defining correlates of protection.

We found that all vaccinated animals that responded effectively to ART as well as four of seven mock-vaccinated macaques controlled viremia after ART suspension. The restriction of viral replication in some of the animals treated with antiretroviral therapy alone was unexpected, as in a previous study only treatment within the first 24 hours from exposure protected macaques from viremia[37]. It is possible, however, that the combination of blunting of acute viremia and long-term ART treatment may have limited the immunological damage in some animals and allowed for an appropriate immunological response to the virus.

Only a few HIV-I-infected individuals have been able to suppress viremia following ART suspension[3–6]. Recent work suggests that HAART treatment during primary HIV-I infection may indeed limit dissemination of the virus, preserve virus-specific $CD4^+$ and $CD8^+$ t cell response, and limit immunological damage[6,13]. Moreover, in one of these studies, 4 HIV-I-infected individuals treated within the first 20 days from infection temporarily contained viremia when HAART was discontinued[6]. Thus, the fact that in our study four of seven macaques treated with ART during primary SIV_{251} infection (group A) also restricted viral replication in the absence of ART mirrors the observation in humans, validates this animal model, and underscores the importance of early diagnosis and treatment of HIV-I infection in humans.

The effect of ART alone in our study has interfered with our ability to reach unequivocal conclusions on the contribution of NYVAC-SIV-*gpe* vaccination to the containment of viremia following ART suspension. Nevertheless, the ability of a NYVAC-based candidate vaccine to induce both $CD4^+$ and $CD8^+$ t cell response in infected animals warrants studies in HAART-treated HIV-I-infected individuals, as vigorous $CD4^+$ and $CD8^+$ t cell responses have been associated with containment of HIV-I infection in humans[6,12–14].

The NYVAC vector, derived from the Copenhagen strain of vaccinia, has been attenuated by precise deletions in 18 open reading frames encoding functions implicated in poxvirus virulence and host-range phenotype[38]. Safety concerns, however, need to be addressed. In our study, inoculation of the NYVAC or NYVAC-SIV vector in SIV-infected animals did not result in any adverse effects. In addition, in a previous unpublished study, we demonstrated the lack of vaccinia dissemination following intravenous, subcutaneous or intramuscular inoculation of 10^8 pfu of NYVAC-SIV recombinant vector in 10 macaques with immunodeficiency ($CD4^+$ t cell count ranging from 60 to 130 and virus load ranging between 10^6 and 10^7 copies of viral RNA per ml of plasma). Although the final safety assessment of the NYVAC vector and therefore usefulness awaits further testing, our study indicates that immune therapy in HIV-I-infected individuals is a rational approach. In addition, our data demonstrate that immune-based intervention likely will be more effective in the presence of ART.

NYVAC and other live-vector vaccine candidates[18–22] that induce t cell-mediated immunity, alone or in conjunction with immunomodulatory molecules that restore immune function and improve the immunogenicity of these vaccines[39–41], may decrease or render intermittent the need for HAART in HIV-I-infected individuals.

Methods

Animals. All animals were colony-bred rhesus macaques (*M. mulatta*) obtained from Covance Research Products (Alice, Texas). The animals were housed and handled in accordance with the standards of the American Association for the Accreditation of Laboratory Animal Care. All animals were in good health, 2–4 y, and weighed 3–6 kg. Before study, all animals were seronegative for SIV, SRV, simian t cell lymphotropic virus type I (STLV-I) and herpesvirus B.

Inoculation and ART. Macaques were inoculated intravenously with 10 $TCID_{50}$ of pathogenic SIV_{251}. At day 15 following inoculation, 16 animals in groups A and B received subcutaneous inoculations of 10 mg/kg/d of PMPA ((R)-9-(2-phosphonylmethoypropyl)adenine; ref. 37), oral administrations of 1.2 mg/kg/d of Stavudine (d4T) divided into 2 doses daily, and intravenous inoculations of 10 mg/kg/d of DDI. At day 28 after infection and thereafter, the daily dose of PMPA was increased to 20 mg/kg/d and Stavudine to 2.4 mg/kg/d.

Viral-load measurement. We quantified SIV_{251} RNA in plasma by nucleic acid sequence-based amplification[42]. Briefly, RNA extracted from plasma was subjected to isothermal amplification with primers specific for SIV_{251} and quantified by electrochemiluminescence chemistry using a coextracted internal standard. The detection limit of this assay was 5×10^2 RNA copies/input volume.

Lymphocyte proliferation assay. Antigen-specific proliferation was measured in PBMC from fresh blood samples. PBMC were isolated by density-gradient centrifugation on Ficoll, resuspended in RPMI-1640 medium (Gibco, BRL) containing 5% human A/B serum (Sigma), and cultured at 10^5 cells/well for 3 d in the absence or in the presence of native purified viral p27 Gag or gp120 proteins (ABL, Rockville, Maryland) or Concanavalin A as a positive control. The cells were then pulsed overnight with 1 μCi of [^{3}H]thymidine before collection. The relative rate of lymphoproliferation was calculated as fold of thymidine incorporation into cellular DNA of cells stimulated with antigens versus cells maintained in media alone (stimulation index, SI). An SI of more than 3 was considered positive.

Detection of Gag 181-tetramer-staining $CD3^+$ $CD8^+$ T lymphocytes. We screened rhesus macaques for the presence of the Mamu-A*01 allele using a PCR-based technique[24]. Freshly prepared PBMC were stained with anti-human CD3 antibodies (CyChrome-labeled, clone SP34, Pharmingen), anti-human CD8αβ antibody (FITC-labeled, Becton-Dickinson) and Mamu-A*01-Gag 181 conjugate (PE-labeled, J. Altman). We analyzed samples on a FACScan (Becton-Dickinson) and the data are presented as percentage of tetramer-positive cells of all $CD3^+$ $CD8^+$ cells, unless specified otherwise. To expand the Gag 181-specific $CD8^+$ t cell population, 5×10^6 cells at 3×10^6 cells/ml were incubated with Gag 181 peptide at a final concentration of 10 μg/ml for 3 d. Recombinant IL-2 (Boehringer) was added at 20 IU/ml and the cells were cultured for an additional 4 d and stained as described for fresh PBMC.

CTL assay. We cultured macaques PBMC overnight in the presence of 100 IU/ml IL-2 and then incubated at different effectors to target cell ratios for 6 hours with ^{51}Cr-labeled autologous transformed B cells pulsed overnight with 1 μg/ml Gag 181. The background killing in the absence of peptide was between 5 and 15% and is subtracted from the values of specific killing. The killing of cells pulsed with unrelated peptide as control was generally similar to the killing observed in the absence of peptide.

Statistical analysis. Comparisons of viral load after initiating ART were made by applying the Wilcoxon rank sum test to the means of the logarithmically transformed RNA copy numbers for each animal. All tests of p27 Gag and gp120 Env levels were performed using repeated measures analysis of variance (ANOVA) on the log-transformed SI. This procedure combines the *t*-tests at each time point while making the necessary correction for the correlations among the multiple values from each animal. The tetramer-positive percentages of $CD3^+$ $CD8^+$ lymphocytes were analyzed using repeated measures ANOVA after the arcsine transformation of the square root of each percentage. This transformation is commonly used with percentage data to bring closer to equality the unequal variances of a range of percentages. ANOVA *P* values are based on the assumption of a normal distribution of the deviations between observed and expected values, and agreement with this assumption was verified. All *P* values reported are two-tailed. The SAS (version 8, SAS Institute, Cary, North Carolina) and StatXact (version 4.0.1, Cytel Software, Cambridge, Massachusetts) statistical software packages were used for these analyses.

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1	02493 0303	Cyrano de Bergerac :	11 SEP 00	*	EP
2	13430 2872	Romeo and Juliet /	11 SEP 00	*	SGC
3	12002 1502	The tragedy of Romeo and Juliet /	11 SEP 00	*	SGC
4	11351 4232	Hiroshima mon amour.	19 SEP 00	*	MML
5	09191 2481	The story of Adele H.	19 SEP 00	*	MML
6	07635 6159	Behavioural ecology :	19 SEP 00	*	SIBL
7	04267 9569	Animal minds /	19 SEP 00	*	SIBL
8	07635 6522	The psychology and behaviour of	19 SEP 00	*	SIBL
9	12870 4075	If a lion could talk :	19 SEP 00	*	SIBL
10	11081 1946	The Fugitive kind	12 SEP 00	*	MML
11	01383 2924	German expressionist drama :	02 OCT 00	*	MML
12	01443 1429	German expressionist drama /	02 OCT 00	*	MML
13	02087 0339	Seascape;	02 OCT 00	*	MML
14	14090 0230	The boy with the Arab strap	16 SEP 00	*	EP
15	10823 4770	The Young Bechet	16 SEP 00	*	EP
16	11272 9633	Stellar regions	16 SEP 00	*	EP
17	12190 7105	Premieres chansons	16 SEP 00	*	EP
18	11272 9609	Duke Ellington & John Coltrane	16 SEP 00	*	EP
19	10416 6232	Hamlet /	16 SEP 00	*	EP
20	10552 3852	Nightmares of nature /	16 SEP 00	*	EP
21	07573 2855	The Biology of sea snakes /	30 SEP 00	*	SGC
22	11351 7367	Three by Tennessee :	30 SEP 00	*	SGC
23	07805 0552	Heroines :	30 SEP 00	*	SGC
24	02435 1187	The serpent's egg,	30 SEP 00	*	SGC
25	01980 3895	A man and a woman;	30 SEP 00	*	SGC
26	07971 7589	The theatre of Tennessee Williams.	30 SEP 00	*	SGC
27	05719 4686	The theatre of Tennessee Williams.	30 SEP 00	*	SGC

Preclinical challenge studies of AIDS vaccines in non-human primates have an important role in the AIDS vaccine development effort. However, to be most useful, challenge models need to be improved to more closely reflect the actual biological circumstances of HIV-1 infection and transmission in humans.

AIDS vaccine models: Challenging challenge viruses

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Animal models are essential in the search for a vaccine to prevent HIV-1 infection. Of these, rhesus macaque models are, by far, the most important. Rhesus monkeys can be experimentally infected with various simian immunodeficiency virus (SIV) strains of differing virulence, many of which cause simian AIDS (refs. 1,2). However, because HIV-1 does not productively infect macaques, it cannot be used as a challenge virus to assess whether a given vaccine can prevent or ameliorate infection[1,2]. Hence, preclinical AIDS vaccine models rarely test the identical vaccine constructs that are planned for human use. Instead, studies in rhesus macaques explore the potential protective efficacy of vaccine concepts, not the actual vaccines being developed for human trials. To increase the relevance of the macaque model to human vaccine trials, hybrid viruses that contain elements of both the SIV and HIV-1 genomes, designated SHIVs (simian/human immunodeficiency viruses), were first engineered several years ago for use as challenge viruses in pre-clinical AIDS vaccine studies[3–6]. In these chimeric viruses, several HIV-1 genes, most commonly *tat*, *rev* and *env*, are substituted for their SIV homologs. One highly virulent SHIV challenge virus, SHIV-89.6P, has been widely used in vaccine experiments, and is usually now considered the 'virus-of-choice'[6,7].

Infection of naive macaques with SHIV-89.6P leads, almost invariably, to a rapid and virtually complete loss of $CD4^+$ T-cells from the peripheral blood and lymphatic tissues[6,7] (Fig. 1). Virus replication proceeds at a rapid and highly reproducible rate, and infected macaques progress very rapidly to AIDS and death. The consistency and rapidity of the virological and immunological outcomes of SHIV-89.6P challenge is useful in vaccine challenge studies, as a statistically significant conclusion can be drawn from a limited number of animals in an acceptably short time-frame[7]. Furthermore, the extreme virulence of SHIV-89.6P infection has been thought to provide a rigorous challenge model for the comparative evaluation of different vaccine concepts. However, SHIV-89.6P differs in important ways from the HIV-1 strains that most commonly infect and are transmitted between humans. In addition, accumulating evidence suggests that despite its exceptional virulence in macaques, SHIV-89.6P may be a paradoxically easy virus for the immune system to control. In other words, SHIV-89.6P may be a sheep in wolf's clothing, with the potential to lead the AIDS vaccine development effort down the wrong path.

The first SHIVs were constructed when the goal of vaccine development was the outright prevention of HIV-1 infection ('sterilizing immunity') via vaccine-induced neutralizing antibodies against HIV-1 Env. Although vaccines able to induce effective neutralizing antibody responses have not yet been developed, *env* is still the primary HIV-1 gene included in SHIVs (refs. 1–6). Perhaps by force of habit, this has remained so even when the vaccine approaches being evaluated do not include HIV-1 Env, and are often predicated on the induction of antiviral $CD8^+$ T-cell responses rather than neutralizing antibodies. The belief that sterilizing immunity to HIV-1 is an attainable goal has waned recently, and the goal of vaccine development has shifted to the containment of virus replication post-infection (primarily by vaccination-induced antiviral $CD8^+$ T-cell responses)[8,9]. A vaccine that allows a substantial containment of viremia in vaccinated individuals who become infected would represent a significant advance, as diminished virus replication is associated with substantially decreased rates of disease progression and HIV-1 transmission[10,11].

The utility of the SHIV models is obvious, yet their limitations must also be considered. Some limitations are inherent to all animal models of human infections; there may be species-specific, overt or subtle differences in how retroviruses are transmitted, or in the way they cause disease[1,2]. However, other limitations can be specific to certain challenge viruses. For instance, a virus that replicates very poorly in a host may be misleadingly easy to protect against by vaccination, and is of little value for studies of viral pathogenesis. Conversely, vaccine studies that employ particularly virulent viruses or experimental challenges delivered at high doses via intravenous inoculation may underestimate the potential protective efficacy of some vaccine strategies.

The prototypes of genetically engineered SHIVs usually replicated poorly when first inoculated into macaques, and were non-pathogenic[1–6]. Substantially higher replication and greatly enhanced pathogenicity were then achieved by serial passage of the SHIVs through naive macaques[1,2,4,7]. The resulting SHIV variants, exemplified by SHIV-89.6P, mutated to cause a rapid, nearly complete depletion of the total $CD4^+$ T-cell population in newly infected macaques. The acquired ability to extensively destroy $CD4^+$ T cells is associated with an increased membrane-fusing capacity of the envelope glycoproteins[12].

Many vaccine immunogens, alone or in combination, have now been evaluated in the SHIV-89.6P model, including naked DNA, recombinant viruses, subunit proteins and peptides[8,9,13–19]. Despite the diversity of immunogens, a surprisingly consistent outcome has been seen in multiple studies: the vaccinated animals become persistently infected with SHIV-89.6P, but the rapid and complete decline in peripheral $CD4^+$ T cells does not occur[8,9,13–19]. Instead, the animals maintain near-normal $CD4^+$ T-cell counts, and have greatly reduced viremia compared to unvaccinated controls. These results have been greeted with substantial enthusiasm, because the 'successfully' vaccinated animals can control virus replication at levels that are, in HIV-1-infected

humans, associated with significantly lowered disease progression and transmission rates—probably the most attainable near-term goals of AIDS vaccine research[6–11].

In HIV-1-infected humans, a disease course like that following SHIV-89.6P challenge of macaques is not unprecedented, but is extremely rare[20,21] (Fig. 1). A fear, therefore, is that because the rapid destruction of CD4⁺ T-cells caused by SHIV-89.6P is abnormal, so might be the nature of the protection achieved against this decline induced by such a variety of vaccine antigens.

We have three interrelated concerns about whether SHIV-89.6P, despite its logistical advantages, should be considered as the virus-of-choice in pre-clinical AIDS vaccine studies. First, although the HIV-1 *env* included in SHIV-89.6P was derived from a 'dual-tropic' virus that used both the CCR5 and CXCR4 coreceptors for entry into macaque CD4⁺ T-cells, sequential passage in macaques has selected for a SHIV that uses only CXCR4; SHIV-89.6P is a 'pure X4 virus'[22]. Primary infection of humans with viruses that use CXCR4, either alone or as well as CCR5, is rare, but when this occurs, a rapid, irreversible loss of peripheral CD4⁺ T-cells is observed[23]. An accelerated loss of CD4⁺ T cells also ensues whenever CXCR4-using viruses emerge during chronic HIV-1 infection, probably due to their enhanced membrane-fusing capacity and their tropism expansion to target naive CD4⁺ T-cells in the thymus and periphery[23,24]. However, most HIV-1 strains transmitted between humans use only CCR5 as their coreceptor (R5 viruses), and they do not cause a rapid, irreversible loss of peripheral CD4⁺ T-cells during primary infection[23–25]. Nor does the latter happen when macaques are infected with an R5 SHIV such as SHIV-162P (ref. 26). Overall, X4 viruses and R5 viruses interact with the primate immune system in significantly different ways, yet it is against the transmission and local, early amplification of R5 viruses that an effective HIV-1 vaccine must protect[9,25,26].

Our second concern relates to the central role of CD4⁺ T-helper cells in determining the outcome of the initial battle between HIV-1 replication and the immune system[27]. Studies in both humans and macaques show that allowing CD4⁺ T-cell help to mature by suppressing viral replication during primary infection can be highly beneficial to the subsequent course of disease[27–30]. Conversely, a virus that replicates with unusual rapidity may destroy too many T-helper cells before they can coordinate both antiviral B cell and CD8⁺ T cell–mediated immunity. Such an outcome has a catastrophic effect on the immune response. Indeed, the loss of T-cell help is so striking that humans and macaques with extremely rapidly progressing HIV-1 or SHIV-89.6P infection fail to seroconvert to viral antigens[6,7,12,20,21]. The temporal development of partially effective adaptive immunity and the destruction of the helper T cells that coordinate these responses can be finely balanced[30,31]. A very early, vaccine-elicited memory response to viral antigens might restrict the initial replication of SHIV-89.6P just sufficiently to preserve enough of the CD4⁺ T-helper pool during the early, critical generative stages of the antiviral response. Protection might arise by priming of CD4⁺ T cells to provide help for early production of neutralizing antibodies by B cells, and/or via activation of antiviral CD8⁺ T cells to limit early expansion in the number of virus-infected cells. Whatever the actual nature of the 'protective' immune effectors raised by various vaccine approaches, their magnitude would be amplified after infection via exposure to viral antigens produced during challenge virus replication. The surviving CD4⁺ T-helper cells could then coordinate effector CD8⁺ T-cell and B-cell memory responses that contain virus replication.

Thirdly, and perhaps most importantly, SHIV-89.6P is unusually sensitive to autologous neutralizing antibodies[6,32]. If prior vaccination enables a partial preservation of CD4⁺ T cells following challenge, sufficient help would then be available for B cells to mount an anamnestic neutralizing antibody response directed against the SHIV-89.6P Env. Given the sensitivity of SHIV-89.6P to autologous neutralizing antibodies, it may be relatively easy to suppress virus replication, and thereby protect against the rapid decline in CD4⁺ T cells and rapid disease progression that typically follows SHIV-89.6P infection. However, this dynamic is not

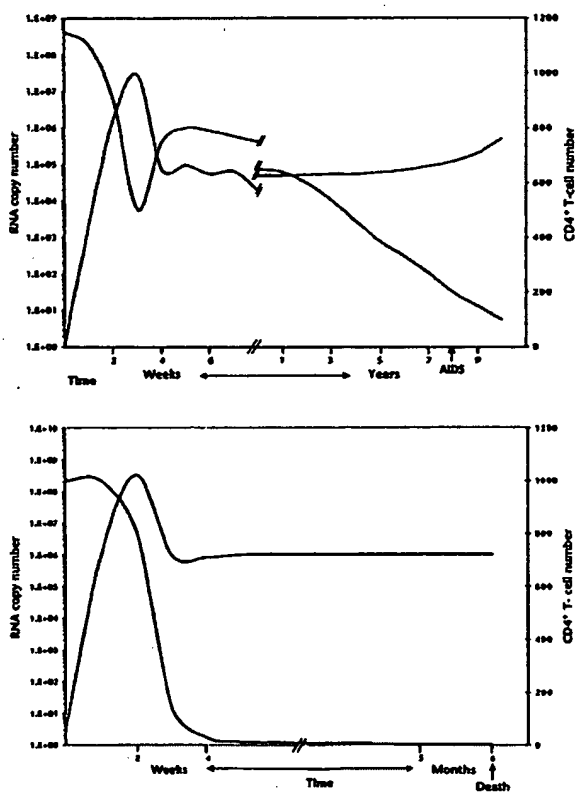

Fig. 1 Contrasting natural histories of AIDS virus infections. HIV-1 infection of humans results in highly variable rates of disease progression that are associated with set-point levels of virus replication and rates of decline of peripheral CD4⁺ T cells. A schematized typical course of progression to AIDS following initial HIV-1 infection is shown, characterized by chronic intermediate levels of plasma viremia, a steady loss of peripheral CD4⁺ T cells, and the development of AIDS after 7–10 years (above). In contrast, SHIV-89.6P infection of rhesus macaques results in near-complete destruction of the peripheral CD4⁺ T-cell population within the first weeks of infection, uncontrolled high-level virus replication, and rapid progression to AIDS and death (below).

characteristic of typical HIV-1 infection and the subsequent immune response in humans. In infected humans, HIV-1-specific CD4⁺ T cells are rarely acutely depleted to the extent that antiviral immune responses cannot be generated[20,21,24]. Moreover, most primary HIV-1 isolates resist antibody neutralization, and only rarely can the combined activity of antiviral CD4⁺ and CD8⁺ T-cell responses durably keep virus replication at low levels[10,24,27].

We believe some of the vaccines that have protected macaques against SHIV-89.6P infection represent important concepts that should be expedited into clinical trials in humans. But the protection elicited by other vaccines may be artifacts of the SHIV-89.6P model, and irrelevant to protecting humans against HIV-1. Few vaccine strategies have been evaluated for protective efficacy using both SHIV-89.6P and alternative pathogenic SIV challenge models. For those that have, the results obtained in the SHIV-89.6P model have always been the more impressive[13,14,33,34] (and D. Watkins, pers. comm.). Indeed, marked and durable control of a pathogenic SIV has never been achieved by vaccination, other than by a live-attenuated vaccine[1,2,8,9]. We therefore suggest that vaccine concepts that have given positive results in the SHIV-89.6P model should be retested against an RS virus that does not cause an abnormally rapid and profound loss of CD4⁺ T-cells (such as SHIV-162P, SIVsmE660, SIVmac251 or SIVmac239). Furthermore, given the tremendous genetic diversity of HIV-1 worldwide, it is essential that the ability of a given vaccine strategy to protect against genetically distinct virus challenges be rigorously investigated in macaque models. So far, all of the experimental SHIV challenge studies have employed vaccine immunogens that are genetically identical (or nearly so) to the challenge viruses[13–18]. The breadth of protective efficacy that can be engendered by vaccination has yet to be addressed experimentally in macaques, yet a single amino-acid change in Gag is reported to be sufficient to enable SHIV-89.6P to escape from immune containment[35]. Given that all of the SHIVs currently used in vaccine studies employ a 'backbone' of SIV genes derived from a single molecular clone (SIVmac239), it will be necessary to develop immunogens that differ from the challenge virus across the viral genome, and not just in the *tat*, *rev* and *env* genes that represent the HIV-1 component of the SHIV.

Animal models are an essential resource for evaluating the safety and comparative immunogenicity of candidate AIDS vaccine strategies in preparation for Phase I human studies. Some vaccine concepts should be rejected before Phase I human studies begin, if a similar concept is so obviously superior in a macaque model. However, if protection from the acutely pathogenic effects of SHIV-89.6P is the only requirement to move a vaccine concept into Phase I human trials, the evaluation pipeline for AIDS vaccines may be over-burdened by vaccines of widely varying immunogenicity and plausibility.

Animal models cannot determine whether a vaccine will be effective against HIV-1 infection of humans; only Phase III trials in humans can do so. However, challenge experiments in the macaque models can potentially add important insights to those gained in Phase I and II studies in humans, and they should meaningfully inform decisions that will impact many thousands of volunteers and involve many millions of dollars. To be most informative and helpful, challenge studies in macaques should now seek to resolve the most difficult issues in AIDS vaccine development, and, depending on the nature of the scientific question under evaluation, studies may need to use more than a single challenge virus. Within this context, it is essential that macaque models be improved so as to mimic, as closely as possible, the actual circumstances of HIV-1 infection and transmission in humans.

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Short communication

Effector cytotoxic T lymphocyte numbers induced by vaccination should exceed levels in chronic infection for protection from HIV

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Abstract

Recent technological advances have revolutionised our capacity to induce cytotoxic T lymphocyte (CTL) responses with a variety of vaccine formulations and delivery systems. However, the conditions required for a CTL-inducing vaccine to provide protection from infection or disease are poorly understood, and the results of challenge experiments have not been consistent. Here we use a mathematical model to examine the requirements necessary for successful vaccination against human immunodeficiency virus (HIV) through cellular immunity. We describe the interaction between cytotoxic T cells and infected lymphocytes, capturing the essence of a persistent infection of immune cells. We conclude that to protect from infection, the cellular immune response should be boosted to levels exceeding those in chronic infection. This requires either that effector CTL exceed this threshold before infection, or that a memory CTL population is established that can yield this level of effector CTL very quickly upon infection.

Keywords: Persistent infection; Cellular immunity; Prophylactic vaccine

1. Introduction

Cytotoxic T lymphocytes (CTL) are key effectors in mediating protective immunity against immunodeficiency viruses [1–5]. CTL responses can be readily induced [6–9], but the inconsistent results of challenge experiments [7,10–22] raise questions concerning the correlates of protective immunity. Here we use a mathematical model to clarify the requirements necessary for successful prophylactic vaccination against human immunodeficiency virus (HIV) through cellular immunity. To this end, we describe the interaction between cytotoxic T cells, uninfected and infected lymphocytes, capturing the essence of a persistent infection of immune cells.

The model used to describe the infection process is the basic tool for the study of in vivo HIV dynamics [23–26]. A schematic representation is given in Fig. 1.

This system can be described with the following differential equations:

$$\frac{dT}{dt} = \lambda - \delta_T T - \beta IT, \qquad \frac{dI}{dt} = \beta IT - \delta_I I - kEI,$$
$$\frac{dE}{dt} = aEI - \delta_E E \tag{1}$$

2. CTL threshold for protection

A prophylactic vaccine should prevent primary infection by controlling viral growth, thereby avoiding a rise in viremia after exposure to HIV. In other words, a small number of cells might become infected upon exposure, but if the virus-specific CTL elicited by the vaccine suppress the growth of the virus population, the number of infected cells subsequently will not increase. For a vaccine to be successful, it must induce a specific immune response exceeding a certain threshold [27]. We used the above model to predict the number of virus-specific CTL required to prevent infection with HIV.

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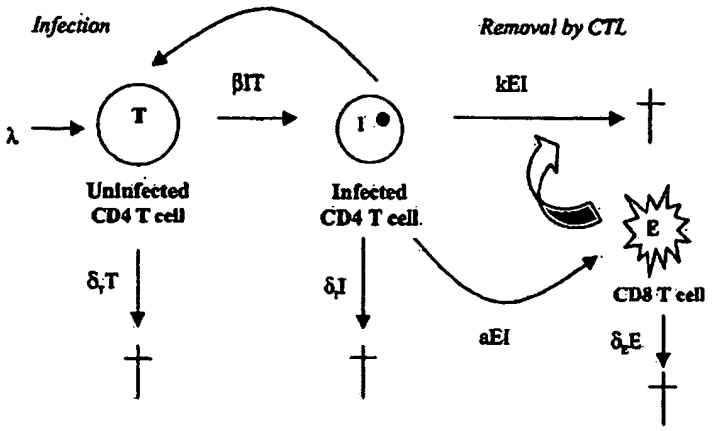

Fig. 1. Schematic representation of the model of HIV infection used. CD4+ T cells are produced at rate λ and die at a rate $\delta_T T$. Hence, their average lifetime is $1/\delta_T$. CD4+ T cells can be infected by free virus. The rate of infection is proportional to the number of uninfected CD4+ T cells and the number of infected cells I, so CD4+ T cells are infected at rate βIT. Infected cells have an average lifetime of $1/\delta_I$, so die at rate $\delta_I I$. Cytotoxic T lymphocytes (CTL, represented by variable E) lyse infected cells at rate k, leading to a death rate of infected cells by CTL lysis kEI. CTL can proliferate after activation by the infected cells: the CTL population grows at rate aEI. CTL have an average life span of $1/\delta_E$, so they die at a rate $\delta_E E$. The assumptions made all favour the possibility that a CTL-inducing vaccine is a biologically feasible approach. We assumed that the CTL response successfully targets the infection virus, that HIV-infected cells are uniformly susceptible to killing by CTL, and that CTL are qualitatively uniform and unaffected by antigen encounter.

To calculate this threshold we used the notion of basic reproductive number (R_0) [28], the number of cells infected by a single cell in a susceptible cell population (see Appendix A). If R_0 of the infecting virus is smaller than 1, the infection cannot spread within an individual [29]. In the chronic phase of HIV infection, the immune response just manages to keep the viral population at bay. Each infected cell then yields by definition on average one infected cell during its lifetime. To prevent the virus population from growing in a newly infected individual, a vaccine should reduce the average number of newly infected cells per infected cell below 1. Increasing the number of CTL reduces the virus basic reproductive number. In a healthy individual, the number of target cells is higher than in a chronically infected patient, raising the basic reproductive number of an infecting virus. To keep the virus basic reproductive number below 1, the number of CTL effectors should be higher than in post-acute infection (see Appendix A). This result is robust: whatever the mechanism of activation and proliferation of effector T cells in response to antigen, the threshold of CTL as formulated above remains the same. It is an important observation: a successful vaccine should boost CTL to levels exceeding those in chronic infection. If the immune system is incapable of clearing the infection under normal circumstances, it is unlikely that a single vaccination will cause lasting protection.

Achieving these levels of CTL at infection is not a full guarantee for protection. An additional requirement is that CTL exceed this level as long as virus can potentially replicate. If upon infection, CTL are above the threshold, virus will not grow and cannot stimulate the CTL to proliferate. As antigenic stimulation declines, CTL numbers will drop over time, until they reach the threshold level. If at that stage virus is still present at significant levels, the viral population can grow and infection may follow. The exact number of CTL needed to guarantee clearance will depend on what virus titre is associated with viral clearance, on the number of CTL present at the moment of infection, and on the rates of decline of CTL and infected cells.

3. Implications

Vaccination should lead to a level of CTL effectors above the threshold at the moment of infection, or to the establishment of a CTL precursor population that would allow the anamnestic response to rapidly reach this threshold number on subsequent exposure to virus. In the first scenario, it is improbable that such CTL levels could be maintained without a vaccine delivery system that provides a constant source of antigenic stimulation. In the second scenario, experimental data describing the speed with which the threshold must be reached to prevent the establishment of persistent infection is needed. The magnitude of the peak of the CTL response after challenge, in terms of percentage CD8 cells specific for SIV Gag p11C, can be greatly increased by vaccination, reaching 40% [10]. However, the timing of the peak is the same in vaccinated and control animals, and virus is not cleared. In two other experiments, vaccination leads to a higher and earlier post-challenge peak of CTL effectors but does not protect the animals from infection, probably because the response does not peak early enough [30,31]. It is likely that a narrow kinetic window, which will depend on a variety of biological factors, applies. For example, if persistent infection ensues at a certain virus threshold, then

the speed with which the effector threshold must be reached will increase with inoculation dose.

Stimulating the CD4 T helper response is a possible way to increase the efficiency of the primary and secondary CTL response. However, enhancing the CD4 T helper response may present a problem in the context of HIV infection as this provides more target cells for infection — this is discussed separately [32]. We have not taken into account possible differences in CTL efficiency in our model because it is presently unclear how CTL exactly they differ. Homing properties are likely determinants of efficiency, as CTL that reach antigen probably expand preferentially. Mucosally delivered vaccines may be more effective at inducing these CTL populations, suggesting such vaccines may be better candidates [33,34].

Vaccination-challenge experiments should attempt to identify the factors that govern the kinetics of the CTL response to challenge, in order to determine more consistent predictors of biological outcome. Our observations indicate that alternative vaccination strategies would potentially be more promising, either alone or combined with approaches enhancing other effector arms of the immune response.

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Appendix A

R_0 can be expressed as the average life span of an infected cell, multiplied by the number of target cells infected during its lifetime:

$$R_0 = \frac{\beta T_{\text{uninf}}}{\delta_I + kE_{\text{uninf}}} \tag{A.1}$$

where T_{uninf} and E_{uninf} are target cell and CTL numbers in an uninfected individual. If the number of effector cells E_{uninf} is sufficiently high, the basic reproductive number will be lower than 1. The threshold number of CTL can be found by solving $R_0 = 1$, to find:

$$E_{\text{threshold}} = \frac{\beta T_{\text{uninf}} - \delta_I}{k} \tag{A.2}$$

where $E_{\text{threshold}}$ is the threshold level of CTL above which control of virus growth is possible.

In the chronic phase of infection, the viremia stabilises at a set point until the patient develops AIDS. As the viral population does not grow in size, we may assume that $R_0 \approx 1$. Consequently, target cell numbers stabilise at the following level:

$$T_{\text{chronic}} = \frac{\delta_I + kE_{\text{chronic}}}{\beta} \tag{A.3}$$

where E_{chronic} is the number of CTL during chronic infection. Using expressions (A.2) and (A.3), we can now rewrite the CTL threshold as

$$E_{\text{threshold}} = \left(\frac{T_{\text{uninf}}}{T_{\text{chronic}}}\right) E_{\text{chronic}} + \frac{\delta_I}{k}\left(\frac{T_{\text{uninf}}}{T_{\text{chronic}}} - 1\right) \tag{A.4}$$

where δ_I, k and β are constant from primary infection to chronic infection.

This threshold depends on the ratio of target cells in a healthy individual to target cells in chronic infection ($T_{\text{uninf}}/T_{\text{chronic}}$). This ratio normally exceeds 1: in any given individual, target cell numbers are reduced in chronic HIV infection compared to the healthy state.

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HIV vaccines 1983–2003

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Twenty years after the discovery of HIV, there is still no vaccine. This year, an envelope vaccine aimed at stimulating neutralizing antibodies was unable to protect against infection in phase 3 trials. But more than 20 HIV vaccines designed to stimulate T-cell responses are being developed. Will any of them work?

Earlier this year, the results of the first phase 3 efficacy trial of a vaccine against AIDS were announced (see the VaxGen website; http://www.vaxgen.com). The gp120 vaccine, tested in 5,000 at-risk volunteers, showed no protective effect. This result, although not unexpected, cast some gloom in the vaccine development field and raised fundamental questions: is a vaccine against AIDS possible at all? Will it ever be able to cope with HIV variability? Will it offer sterilizing immunity or only partial protection? Are there alternative approaches to stimulating neutralizing antibodies? Although it is not unusual for the development of a vaccine to take 20 years, this goal still seems a long way off for HIV.

The most essential of these questions is whether a vaccine will be possible at all. Throughout the development of previous vaccines for other viruses, it was clear that people who recovered from acute viral infections were immune from a subsequent attack by the same virus. This is not so for HIV because no one is known to have recovered from, and completely cleared, acute infection. HIV causes a chronic infection with reservoirs of virus in T-cell, macrophage and monocyte compartments, where some of it is integrated as a silent provirus[1]. The virus diversifies during the infection, with repeated selection of mutants that escape both antibody and T-cell immune responses[2,3]. Control of the infection by T cells seems to determine the progression of the infection, and it is possible that some infected people can control infection indefinitely, especially if helped by antiretroviral drugs[4].

A very relevant issue is whether superinfection occurs in infected people who are exposed repeatedly to the virus. This can happen[5], but it is not known how often. If superinfection is rare, it means that the immune response to HIV, although unable to control established infection completely, may be able to increase the threshold for new infections. Support for this conclusion comes from macaques that are chronically infected with attenuated SIV but resist superinfection with more aggressive virus[6,7]. Similarly, macaques that are infected with SIV and then immediately treated briefly with antiretroviral drugs control their infection and resist superinfection[8]; this resistance is abrogated by the removal of $CD8^+$ T cells[9]. Thus, there is some evidence that the immune response can, under certain circumstances, prevent HIV or SIV infection.

At the time that HIV was identified, our understanding of the immune response was relatively poor. Cytotoxic T cells were not known to recognize viral peptides until 1986 (ref. 10), the T-cell receptor had not been discovered and the distinction between T-helper type 1 (T_H1) and T_H2 cells had not been made. Attempts to design an HIV vaccine during this period should therefore be viewed alongside these and other advances in basic immunology. Table 1 shows the principal steps, not all of them forward, in HIV vaccine design in the past 20 years. These steps will be reviewed in greater detail below.

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Antibody immunity

Studies of vaccines that protect macaques against SIV infection indicate that antibody-mediated protection is possible. It has been shown repeatedly that vaccines based on the viral envelope can protect nonhuman primates challenged with homologous virus[11–16]. But the numbers of animals used in such studies are small, and the studies may have limited relevance to humans[17]. It was disconcerting to find that unlike viruses adapted to laboratory culture, primary HIV isolates from infected patients were resistant to neutralization[18]. These isolates were later shown to use different coreceptors[19], although this fact alone does not account for the difficulty in neutralization. Two recent studies have shown that neutralizing antibodies directed at the envelope are made during HIV infection, but as they appear they immediately select for viral escape mutants, thereby becoming irrelevant[20,21].

Sera from individuals infected with HIV have been analyzed extensively for the presence of neutralizing antibodies. Five human monoclonal antibodies have been found that are capable of neutralizing a broad range of primary B-clade HIV isolates[22]. Two of these require CD4 to alter the conformation of gp120; the other three have been characterized in detail. The first antibody binds to the CD4 binding site on the gp120 domain (Fig. 1), but it needs an unusually long complementarity-determining region-3 loop to access the deeply recessed site. The second antibody recognizes a complex polymannose epitope, but it has extraordinary structural features rarely seen on other antibody molecules (D. Burton, personal communication). The third antibody binds to a site on gp41 on the native spike that remains hidden before CD4 binding. These antibodies can protect severe combined immunodeficient mice that have been reconstituted with human lymphoid cells against challenge with HIV[23] and can also protect monkeys against challenge with an SIV/HIV (SHIV) hybrid virus[24–26]. The titers required are high, however, and might be difficult to achieve by active immunization, even if it were possible to devise ways in which to persuade the immune system to generate antibodies of these specificities.

Most antibodies that neutralize can be easily evaded by mutation, and those that bind gp120 monomers seem to be irrelevant. The gp120 crystal structure indicates why neutralization is difficult[27]. The envelope is a trimer of gp120-gp41 heterodimers. The trimer is held together by interactions involving conserved gp120 surfaces that are not exposed on the virion surface but, on gp120 shedding, act as a decoy to stimulate largely irrelevant antibodies. Hypervariable loops mask the critical receptor-binding sites. The exposed surface is covered in asparagine-linked carbohydrates. The importance of these carbohydrates is clear from studies in infected individuals, which show that viral escape is facilitated by changes in glycosylation[20]. Similarly, in macaques, challenge with SIV that has been deliberately mutated to remove glycosylation around the V1 loop results in an effective antibody response that can control the virus, but only until mutations repair the glycosylation deficits[28]. Although not unreasonable at the time, the first vaccines to be tested in the 1980s were unfortunately based on monomeric gp120.

Table 1 Principal steps towards an HIV vaccine since 1983

Year	Event
1983	Recombinant vaccinia virus as a candidate vaccine[103,104]
1984–1996	Identification of HIV cell receptors[105,106]
1990	Envelope-based vaccines protect chimpanzees against homologous HIV challenge[11]
1991	Protection of macaques by inactivated SIV is dependent on cells in which vaccine is grown[31]
1991–2002	HIV/SIV can escape from $CD8^+$ T cells and vaccines[41–46,60]
1992	Attenuated SIV protects against challenge with wild-type SIV[6]
1993	CD4 binding alters conformation of gp120 (ref. 107)
1993	DNA vaccine can protect against viral infection[52]
1995	Resistance of primary HIV isolates to neutralization[18]
1996–2003	Characterization of binding sites for neutralizing monoclonal antibodies[108–110]
1998	Structure of gp120 (ref. 26)
1998	Development of common immunogen prime-boost strategies[58,59,76,77,79]
1999	$CD8^+$ T cells central to controlling acute and chronic SIV infection[111,112]
2000	$CD8^+$ T-cell inducing vaccines protect against SHIV89.6P challenge[55–59]
2003	Virus escapes antibody neutralization *in vivo*[20,21]
2003	First phase 3 vaccine trial completed but no protection observed[93]

Ten years ago, it was claimed that formalin-fixed SIV could effectively protect against SIV challenge in macaques[29,30]. But Stott *et al.*[31] showed that this protection was not virus-specific; in their study, protection was seen only when vaccine virus (before inactivation) and challenge virus were grown in human T-cell lines. Macaques challenged with virus grown in macaque cells were unprotected[31]. SIV and HIV acquire large amounts of major histocompatibility complex (MHC) class I and II molecules, as well as other surface proteins, when they bud from the surface of T cells or macrophages, so the protective immune response might be directed against these acquired human proteins. The original result[31] was regarded as an artifact, although the observed protection was better than any seen in an experimental SIV or SHIV vaccine system. Some attempts have been made to show that similar protection might occur if the virus is grown in cells of a different MHC type, but the results have been inconclusive. The enigma remains and may deserve renewed attention.

Finding vaccines that stimulate antibodies capable of neutralizing primary HIV-1 isolates must still take the highest priority. The challenge is to find ways of inducing such antibodies reliably and in sufficiently high titers, but this may require advances in basic immunology. Structural information should help to find ways of stabilizing envelope proteins in vaccines in conformations that, for example, expose the binding site for the chemokine receptor[32]. Targeting gp41 also may be an option. T20, a peptide that interferes with the hairpin loop formation in gp41 that is necessary for membrane fusion and viral entry, was recently introduced to therapy[33], offering hope for approaches based on a better understanding of the structure-function relationships of viral molecules.

T-cell approaches

Traditionally, the stimulation of a good neutralizing antibody is sufficient for a vaccine. But live attenuated vaccines, which stimulate strong $CD4^+$ and $CD8^+$ T-cell responses and neutralizing antibodies, are more efficient than are inactivated virus or purified protein subunit vaccines, which are poor at stimulating $CD8^+$ T cells. Because the stimulation of neutralizing antibodies is problematic in HIV infection, nearly all current vaccine approaches (Table 2) are aimed at stimulating T-cell

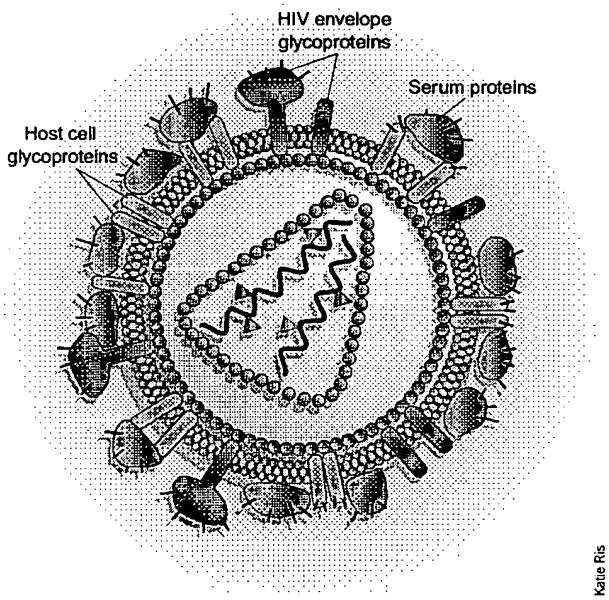

Figure 1 The HIV target for antibodies (not drawn to scale). The textbooks have imprinted into our minds a picture of a perfect HIV virion with a core wrapped in a membrane containing nicely shaped trimeric gp160 spikes on its surface. The reality of what the immune system actually faces is quite different. Thus, only 1 in about 1,000–10,000 HIV particles is not defective and can productively infect host cells, which may, but often does not, leave the relevant antigenic determinants intact. In addition to the HIV envelope spikes (red), HIV particles carry in their membranes numerous host cell–derived glycoproteins (orange) and an array of serum proteins nonspecifically attached to the virion surface (green). Many of the original (approximately) 72 functional spikes have shed their gp120 subunits and may display a conformationally irrelevant postfusion gp41 (red). The remaining intact spikes are highly glycosylated, flexible on the surface and may differ by up to 10% of amino acids between different HIV virions within an individual at a particular time point, thus interfering with the affinity maturation of antibodies.

responses. They are based on the assumption that the induction of a strong $CD8^+$ and $CD4^+$ T-cell response by vaccination will abort or control early HIV infection.

HIV stimulates a strong $CD8^+$ T-cell response during acute viremia and usually persists through the chronic phase of infection[4,34]. A $CD4^+$ T-cell response is also generated early on but is susceptible to damage by the virus, which preferentially infects HIV-specific $CD4^+$ T cells[35–37]. In mice, $CD4^+$ T-cell help is crucial for priming an effective memory $CD8^+$ T-cell response; in mice deficient in $CD4^+$ T cells, pathogen exposure generates normal numbers of memory $CD8^+$ T cells, but these have poor replicative capacity when re-exposed to the microbe[38,39]. In HIV infection, therefore, the initial $CD8^+$ T-cell response may be effective in inducing memory T cells capable of regeneration and a full range of functions, whereas T cells primed later in the infection may be defective even if they are detectable in some assays[40]. Because $CD8^+$ T cells select for HIV escape mutants[41–49], new immune responses are needed during the infection, but if $CD4^+$ T-cell function becomes impaired, these 'secondary' T cells may be less capable of controlling the virus.

Whereas neutralizing antibodies can prevent infection, $CD8^+$ T-cell responses cannot. These cytotoxic T lymphocytes (CTLs) react to other cells of the body that are infected by HIV and present peptide fragments of viral proteins bound to MHC class I proteins[10]. $CD8^+$ T cells kill the infected cells, thereby reducing the production of new HIV virions. They can also inhibit entry of HIV-1 by releasing the β-chemokines RANTES, MIP-1α and MIP-1β, which compete for the CCR5 receptor[50], and other cytokines with antiviral activity. A vaccine should stimulate high numbers of $CD8^+$ T memory cells, which rapidly release cytokines and chemokines on subsequent antigen contact and start killing target cells (Fig. 2). But these cells may need to be expanded to outnumber the virus-infected cells and distributed to several sites around the body. Thus, full antiviral activity may take days to develop and will only control, rather than prevent, viral infection.

Even though $CD8^+$ T cells cannot neutralize virus, there is ample experimental evidence that vaccination to stimulate these T cells can protect mice against high-dose challenges with several viruses[51–54]. Vaccinated mice become infected, but have lower titers of virus as compared with unvaccinated controls. The immune system of the mouse can then cope well with the low amounts of virus and no disease develops.

Recently, vaccines designed to stimulate $CD8^+$ and $CD4^+$ T-cell immunity have protected macaques from challenge with the aggressive strain SHIV89.6P[55–59], which causes a rapid decline in $CD4^+$ T-cell numbers and fatal immunodeficiency. The vaccinated macaques were infected but had a viral load that was 1,000 times less than that in unvaccinated controls. These studies provide the strongest experimental rationale for the current vaccine approach based on $CD8^+$ T cells.

But there are reasons to be cautious. Barouch *et al.*[60] have shown that this SHIV strain can escape vaccine-induced immune control by the mutation of a single amino acid—a process that is facilitated by the focus of the T-cell response on a dominant epitope. If this is not an

Table 2 Prophylactic HIV vaccines in clinical trials

Vaccine	Immunogen	Clade	Sponsor	Country	Phase
AIDSVAX B/E	gp120	B, E	VaxGen	Thailand	3
AIDSVAX B/B	gp120	B	VaxGen	USA	3
ALVAC vCP1452	Env-Gag-Pol-CTL	B	NIAID	USA	2b
AIDSVAX B/B	gp120	B			
ALVAC vCP1452	Env-Gag-Pol-CTL	B	NIAID	Brazil, Haiti, Peru	2b
AIDSVAX B/B	gp120	B		Trinidad & Tobago	
DNA.HIVA	Gag-CTL	A	IAVI/MRC	UK	2a
MVA.HIVA	Gag-CTL	A		Uganda	
ALVAC vCP205 or vCP1452	Env-Gag-Pol-CTL	B	NIAID	USA	2a
AIDSVAX B/B	gp120	B			
ALVAC vCP205	Env-Gag-Pol	B	WRAIR	USA	1
ALVAC vCP1452	Env-Gag-Pol-CTL	B	NIAID	USA	1
DNA.HIVA	Gag-CTL	A	IAVI/MRC	Kenya	1
MVA.HIVA	Gag-CTL	A			
MRKAd5	Gag	B	Merck	USA	1
Poly-env1-vaccinia	Env	A, B, C D, E	St Jude's	USA	1
VCR-HIVDNA009-99-VP	Env	A, B, C	NIAID/VRC	USA	1
	Gag-Pol-Nef	B			
GTU-Nef DNA	Nef	B	FIT Biotech	Finland	1
VCR4302 DNA	Gag-Pol	B	NIAID/VRC	USA	1
Gag DNA	Gag	B	Merck	USA	1
PGA2/JS2 DNA	Gag, RT, Env, Tat, Rev, Vpu	B	NIAID	USA	1
NefTat fusion/gp120	Nef-Tat, gp120	B	NIAID	USA	1
LIPO-4T lipopeptide	Gag-Pol-Nef-TT-CD4	B	ANRS	France	1
ALVAC vCP1452	Env-Gag-Pol-CTL	B	ANRS/Aventis	France	1
LIPO-5T or LIPO 6T lipopeptide	Gag-Pol-Nef-TT-CD4	B			

All trials are being conducted in HIV-negative volunteers at either low risk (phases 1 and 2a) or high risk (phases 2b or 3). CTL denotes CTL epitopes. CD4 denotes T-helper epitopes. ANRS, National Agency for AIDS Research; IAVI, International AIDS Vaccine Initiative; MRC, Medical Research Council of the United Kingdom; NIAID, National Institute for Allergy and Infectious Diseases; VRC, Vaccine Research Center; WRAIR, Walter Reed Army Institute of Research. Table adapted from the International AIDS Vaccine Initiative website (http://www.iavi.org).

isolated incident, such escape could be a real concern when immunity allows low levels of virus to persist. In addition, it might be, paradoxically, relatively easy to protect against SHIV89.6P despite its virulence: it has proved more difficult to protect with similar vaccinations against SIV_{MAC239}, which is possibly closer to HIV in pathogenicity[61]. Such differences need explanation.

The reservations can be tempered by the fact that the dose of HIV encountered in a single exposure during sexual contact in humans is about 100 times less than the dose of SIV typically used to challenge vaccinated macaques. The studies discussed above use high-dose challenges to guarantee that all control macaques are infected. By contrast, many sexual exposures to HIV may be necessary before humans become infected[62,63]; consequently, it may be easier to protect against infection with a vaccine that stimulates T-cell immunity. If humans are repeatedly exposed, however, they will eventually become infected. Vaccination might raise the threshold for infection, reducing the absolute risk of infection from a single exposure to HIV and delaying infection in those who are repeatedly exposed. Those who are infected may have lower amounts of virus, similar to the vaccinated macaques challenged with SHIV89.6P. Although not ideal, these features could offer the benefits of a reduction in primary viremia and viral set point, with slower progression to AIDS and reduced chances of transmitting the virus.

Support for the idea that vaccination might prevent or abort early infection in humans comes from studies of rare individuals who are highly exposed to HIV but remain uninfected for prolonged periods of time; such individuals account for about 5% of the exposed population. $CD8^+$ T-cell responses have been observed in highly exposed but uninfected sex workers and HIV-discordant couples[64,65]. These individuals can also have $CD4^+$ T-cell responses to HIV but no serum antibodies. Whether the T cells are protecting them is uncertain, but direct genetic causes have been so far excluded. In Nairobi, some sex workers became susceptible when they changed their lifestyle, which suggests that immune protection needs continuous antigen exposure[66].

Priming of $CD8^+$ T-cell immune responses by vaccines

The stimulation of $CD4^+$ T-cell responses is relatively easy to achieve: any vaccine that stimulates antibodies will stimulate T-helper cells. As antibody-producing B cells exert their antiviral effect at long range, $CD4^+$ T-helper cells need to act only in lymphoid organs. By contrast, $CD8^+$ T cells are more fastidious. They require antigen-presenting dendritic cells for priming. Their effector function is exerted at short range through contact with infected cells that express peptides derived from viral proteins bound to human leukocyte antigen (HLA) class I molecules. They respond to all viral proteins, with a preference in HIV infection for Gag and Nef[34,67].

Priming of $CD8^+$ T cells is normally achieved by dendritic cells that either are infected or contain reprocessed viral antigen, and that enter lymph nodes to stimulate $CD8^+$ T cells directly. $CD8^+$ T-cell priming in natural viral infections is highly efficient. In acute infections of Epstein-Barr virus (EBV), 40% of blood $CD8^+$ T cells can become specific for a dominant epitope within weeks of first viral contact[68]. This represents nearly 20 cell divisions from the rare EBV-specific naive T cells. The strength of the acute $CD8^+$ T-cell response in HIV infection is smaller, comprising 1–10% of peripheral blood $CD8^+$ T cells, but still represents about 15 divisions from the naive T cells[69–72]. Ideally, experimental vaccines should achieve a similar priming of $CD8^+$ T cells.

In macaques, immunization with plasmid DNA encoding SIV Gag, followed by a boost with recombinant modified vaccinia virus Ankara (MVA; a replication-defective vaccinia virus) expressing SIV Gag, stimulated strong $CD8^+$ T-cell responses comprising up to 20% of T cells to a dominant epitope[59,73,74]. Similar immunity was achieved by a DNA prime and recombinant adenoviral boost[58], or by priming with a combination of DNA and interleukin-2 (ref. 75). Thus, vaccination can achieve early $CD8^+$ T-cell responses that are comparable to natural infection. The problem is that these immunogens do not persist and the T-cell response falls away rapidly as the T cells mature to memory T cells[73].

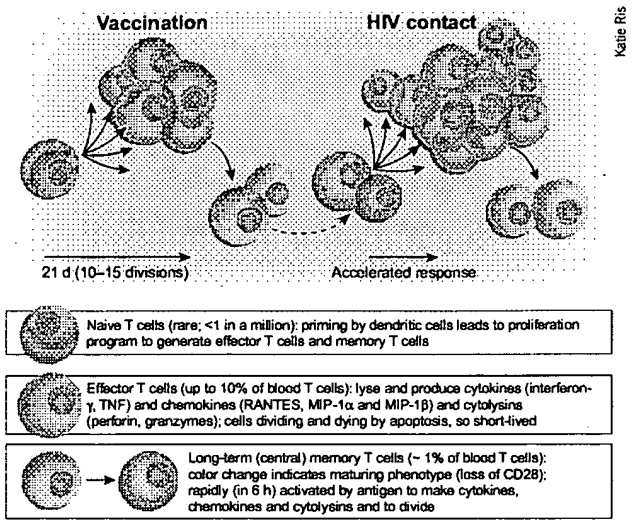

Figure 2 Expansion of $CD8^+$ T cells by vaccination and subsequent response to HIV contact. Shown is the initial expansion of naive cells into effector and memory T cells. Because the vaccine does not persist, the primary immune response is short lived and decays rapidly, leaving memory T cells that further mature over several months. When the memory T cells are exposed to HIV-infected cells, a rapid secondary response ensues.

DNA alone stimulates weak acute $CD8^+$ T-cell responses in macaques, but primes for subsequent responses to a recombinant viral vaccine that are better than the responses to each vaccine alone[58,59,73,74]. The DNA may focus the T cells, ensuring that the same response is boosted after a subsequent immunization with the recombinant virus[76,77]. The virus may have around 200 antigenic proteins; without the priming step, it may not provoke a response to the inserted protein because of immunodominance. Priming and boosting with two vaccines that share a key passenger immunogen is clearly better than using either component alone in mice and macaques, but it has not been confirmed whether this procedure has an advantage over simple priming with recombinant virus in humans (see accompanying review in this issue[78]). DNA may provoke stronger T-cell responses if cytokines such as interleukin-2 are added, either as plasmid DNA or protein[35]. This approach has greatly enhanced $CD8^+$ T-cell responses against SIV Gag in macaques. The use of either adjuvants or other types of immunogens, such as recombinant virus-like particles, to improve responses to DNA also might be useful.

Many viral vectors are being developed as recombinant HIV vaccines, including fowlpox[79], canarypox[80,81], replication-deficient adenovirus-5 (ref. 58), Semliki Forest virus[82] and Venezuelan equine encephalitis virus[83]. Although each may stimulate similar immune responses, they provide opportunities for prime-boost strategies. In some cases, however, pre-existing immunity to the viral vector may limit its usefulness. In addition to the vectors mentioned above, vectors that can persist in the host, such as adeno-associated virus[84], are under consideration. Although the resulting prolonged T-cell responses would be desirable, the consequences of chronic or repeated exposure to foreign antigens on

the immune responses will need to be evaluated carefully. Recombinant bacterial vectors are also being developed as HIV vaccines, including bacillus Calmette-Guérin (BCG)[85] and salmonella[86,87]. BCG has the advantages that it is already known to stimulate T-cell immunity and can be given to newborn babies.

Design of HIV-derived immunogens

There are many possible designs for an HIV-derived immunogen. Gag is usually included because it seems to be most immunogenic in HIV-infected individuals and contains important helper epitopes[34,67]. Similarly, Nef is often part of the construct, although its gene is quite variable and needs to be inactivated for safety reasons when inserted into cells *in vivo*. Reverse transcriptase is conserved but should also be inactivated, perhaps by 'scrambling', because there are conserved epitopes across the active site that should be preserved. Env is also often a component of the formulation, even though it is the most variable protein. In the absence of an efficient strategy for inducing neutralizing antibodies, Env is often included to generate T-cell responses, but there is an argument that it should be left out to leave a 'gap' for the later addition of a neutralizing antibody–inducing immunogen, when one becomes available. Decisions have to be made about what sequence to use; synthetic genes offer the possibility of optimizing amino acid codons to enhance expression in human cells and of using consensus or ancestral sequences[88].

The size of the construct may also be important. The trend is towards making polyprotein vaccines. The T-cell immune response to a fixed-sequence immunogen tends, however, to focus on small numbers of immunodominant epitopes. Thus, a polyprotein vaccine may not necessarily stimulate a broader T-cell response compared with a single protein vaccine. It may be better to break the vaccine up into smaller separate components, thereby forcing the immune response to treat each as a separate invading antigen against which to react.

An important issue is how closely to match the virus and the vaccine[89,90]. This argument generally revolves around clades—for example, is a clade C vaccine needed for South Africa? Intuitively, it seems preferable to match the virus and vaccine as closely as possible, even if the gains are small. The clades differ by 10% to more than 25%, depending on the viral protein. $CD8^+$ T cells respond to peptide fragments of 8–11 amino acids bound to HLA class I proteins, so that on average there is more than one amino acid change per epitope among clades. In an epitope, two-thirds of the amino acid side chains are involved in specific interactions, either with the presenting HLA molecule or with the T-cell receptor (reviewed in ref. 89). As these interactions are very sensitive to change[91], in theory there is only a one in three chance that the T-cell response stimulated by a vaccine of one clade will recognize the epitope from another clade. This problem can be offset if there is a multiepitope response; for example, a five-epitope response to the 'wrong' clade should have an 85% chance or greater of cross-reacting with another clade, but a third of those responses would be to only one epitope.

The danger of too narrow a response is that escape mutants could be easily selected for by the vaccine, particularly if protection is incomplete. This has been observed in macaques vaccinated with DNA for SIV Gag and then challenged with SHIV89.6P (ref. 60). This problem could be a formidable obstacle for T-cell-inducing vaccines: even if the clades of the vaccine and circulating virus are matched, the variability of sequence within a clade (4–10%) may produce similar problems unless the tendency of the T-cell response to focus on immunodominant epitopes is overcome.

Early trials of T-cell vaccines in humans

Several HIV vaccines have entered phase 1 and 2 clinical trials in uninfected volunteers in the United States, Europe, Uganda and Kenya (Table 2). The vaccines include HIV-derived immunogens as adjuvant-associated peptides and proteins; as DNA in plasmid form[90,92]; and as inserts in recombinant canarypox[93], MVA (ref. 94 and M. Mwau *et al.*, unpublished data) and adenovirus (see E.A. Emini: http://63.126.3.84/2002/prelim.htm). So far the immune responses have been small as compared with responses in macaques to the same vaccines, possibly because the doses used are lower and the assays are different. But it is clear that these constructs are immunogenic and that improvement must be possible, for example, by increasing the dose and number of immunizations or by testing different routes of immunization. Combinations of these vaccines in prime-boost approaches may show additive effects. It is too early to say how broad and long-lasting the T-cell responses are in these early trials, but such data will undoubtedly be obtained over the next year.

The assays used to measure immune responses also need research. Currently, enzyme-linked immunospot assays—in which the T cells that make interferon-γ on peptide challenge are counted[95]—are the standard. Although the assay is robust and reliable, it may have limitations when used to measure relatively weak acute T-cell responses. Most of the validation for this assay has been done on well-established, large T-cell responses to EBV, cytomegalovirus or HIV in chronically infected people[96–98], whereas early vaccine-induced responses are likely to be weaker and more fragile. As results start coming in, it will be possible to validate the assays on vaccine-induced responses and improve them. Interferon-γ may not be the best cytokine to measure, given that it has little anti-HIV effect[99]. The use of flow cytometry to measure intracellular cytokine production in T cells stimulated with peptide or antigen *in vitro* might be a better option[100]. This is potentially more sensitive, and additional data on phenotypes of T cells can be gained.

Because most exposures to HIV in vaccine recipients will occur many months after vaccination, the most important measurement will be to quantify long-term memory T cells, especially their proliferative and functional potential[38,39]. The duration of such memory is important[101]. Experiments in mice indicate that memory can be maintained without further antigenic stimulation[102], and Amara *et al.*[59] have seen protection in their immunized macaques seven months after the last vaccination. Protection may be better, however, if the T cells are in a partially or wholly activated state[53]. The apparent necessity for continuous exposure to antigen to maintain protection for the sex workers discussed above suggests that this might be the case[66].

Challenges ahead

There are three main challenges to developing an effective HIV vaccine. The first is to find a vaccine that can stimulate the equivalents of the five known monoclonal neutralizing antibodies in high titers in most or all individuals who are immunized. This may require conceptual breakthroughs in protein engineering and an understanding of how predetermined B cells can be preferentially stimulated and selected. Identifying more monoclonal antibodies that react with other broadly neutralizing epitopes on gp120 and gp41 would also be invaluable.

The second challenge is to find a way to optimize the T-cell-inducing vaccines so that some of them can be taken into phase 2 and phase 3 trials in high-risk volunteers. Studies of $CD8^+$ T-cell-inducing vaccines in animals[51–59] provide real hope that this approach can work, but the difficulties will be formidable. The current crop of vaccines need to be improved to generate bigger responses. Combinations in prime-boost regimes should increase the T-cell responses[59,76,77,79], as should the use of adjuvants and cytokines. Some viral or bacterial vectors may prove to be superior, although it is likely that the current replication-defective vectors will be roughly equivalent. The vaccine will have to stimulate a

long-term memory T-cell response that is broad enough to cope with variability within clades.

The third challenge is to increase the capacity to carry out phase 3 trials in developing countries. These trials will need to be designed so that viral infection, or seroconversion, is the primary end point and reduced viral load is the secondary end point, and an agreed measure of success will have to be decided beforehand. In addition, it must be recognized that finding a useful protective vaccine may take several phase 3 trials with gradually increasing efficacy, as opposed to being realized in a single trial. Those who are testing vaccines should be prepared to mix vaccines and, if necessary, to share intellectual property. A major step forward might be the combination of a T-cell vaccine and a good antibody-stimulating vaccine.

Finally, there are manufacturing issues. Ideally, vaccines should be tested in phase 3 trials only if it will be possible to manufacture them in quantities sufficient to immunize tens of millions of people. But it may be worth taking the first candidates through trials more rapidly to establish that a vaccine can indeed protect humans against HIV. All of this will require huge commitment and very large-scale international collaborations. There are signs that this will be possible.

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Predictive Value of Primate Models for AIDS

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Abstract

A number of obstacles remain in the search for an animal model for HIV infection and pathogenesis that can serve to predict efficacy in humans. HIV-1 fails to replicate and cause disease except in humans or chimpanzees, thereby limiting our ability to evaluate compounds or vaccines prior to human testing. Despite this limitation, nonhuman primate lentivirus models have been established that recapitulate the modes of infection, disease course, and antiviral immunity that is seen in HIV infection of humans. These models have been utilized to understand key aspects of disease and to evaluate concepts in therapies and vaccine development. By necessity, animal models can only be validated after successful trials in humans and the determination of correlates of protection. Because the only vaccine product tested in phase III trials in humans failed to achieve the desired protective threshold, we are as yet unable to validate any of the currently used nonhuman primate models for vaccine research. In the absence of a validated model, many experts in the field have concluded that prophylactic vaccines and therapeutic concepts should bypass primate models, and rely solely upon the systematic testing of each individual and combined vaccine element in human phase I or I/II trials to determine their relative merits. Indeed, a large effort is underway to expand efforts to test all products as part of an international effort termed "The HIV Vaccine Enterprise", with major contributions from the Bill and Melinda Gates Foundation. This Herculean task could potentially be reduced if it were possible to utilize even partially validated nonhuman primate models as part of the screening efforts. The purpose of this article is to review the data from nonhuman primate models that have contributed to our understanding of lentivirus infection and pathogenesis, and to critically evaluate how well these models have predicted outcomes in humans. Key features of the models developed to date are described and their contributions to HIV pathogenesis, therapeutics, and vaccines, are compared. This analysis shows that many of the models at hand have yielded data on drug action and immune responses to vaccines that are congruent with clinical data. This finding suggests that primate models are valuable as adjunctive testing systems to prioritize future therapeutic and vaccine strategies. Nonhuman primate testing of vaccine approaches in particular has provided valuable information and can significantly enhance and accelerate the evaluation of novel concepts necessary to achieve acceptable levels of efficacy. Because major gaps remain in the quest for fully effective vaccines and therapies, it seems prudent to continue aggressive research programs in the nonhuman primate models. (AIDS Reviews 2004;6:187-98)

Key words

Nonhuman primate models. Vaccines. Therapies. Pathogenesis.

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Introduction

Nonhuman primate models for AIDS research have been a major focus of vaccine and pathogenesis work since the discovery of simian AIDS, following accidental cross-species transmission of SIV in the primate centers in the early 1980s[1,2]. Since that time, a great deal of progress has been made in understanding primate lentiviruses in their natural hosts and in their new hosts, with the goal of developing nonhuman models for HIV replication and pathogenesis[3]. Lack of a readily available host, and failure to progress to disease before 10 years, has limited the use of HIV-1 in nonhuman primates[4]. Divergence in protein sequences, and differences in antigenicity between HIV-1 and HIV-2/SIV, required that many therapies and vaccines be tailored specifically for the two lineages. Serial in vivo passage of some human HIV-2 isolates in baboons and in macaques was shown to "heat up" the virus and result in depletion of CD4+ T-cells, a hallmark of AIDS in humans[5-7]. Subsequently, chimeric SHIV viruses bearing HIV envelope genes in a backbone of the SIV genome have been developed[8]; these have been similarly passaged in vivo to achieve high levels of replication and reproducible CD4+ T-cell decline and pathogenesis[9-11]. The ability to recapitulate Koch's postulate with viral mutants has allowed the unequivocal identification of genes that are key for pathogenesis, routes of infection, and targets for drugs and therapies. Using these chimeric SHIVs, several groups have shown that human HIV Env-directed monoclonal antibodies, capable of blocking infection in rodent models[12], were also effective in primates[13-15].

Several excellent reviews of progress in HIV pathogenesis, therapies, and vaccine development, have been written in recent years that point to the use of nonhuman primate models to evaluate concepts and types of compounds[16,17] and vaccines[18-20]. By necessity, animal models can only be validated after successful trials in humans, and correlates of protective immunity may then be identified. The recently completed phase III trials, testing a recombinant gp120 subunit in humans, failed to achieve the desired protective threshold[21]. The HIV Vaccine Enterprise, with major funding from the Bill and Melinda Gates Foundation, has the goal of mounting a concerted effort to develop and test, in parallel, multiple vaccine candidates in humans[22]. The HIV vaccine field faces years, perhaps decades, of clinical evaluation of vaccine candidates. If it were possible to utilize even partially validated nonhuman primate models as part of the screening efforts, then this timeline might be significantly reduced. The purpose of this article is to review the data from nonhuman primate models that have contributed to our understanding of lentivirus infection, pathogenesis, and antiviral immunity, and to critically evaluate how well these models have predicted outcomes for therapies and vaccines in humans.

Current models: pathogenesis and immunology

Animal models have been utilized as tools for understanding elements of infection and pathogenesis for many infectious diseases. Despite concerted efforts, there is as yet no reproducible HIV-1 infection and pathogenesis model other than the chimpanzee[4,23]. The discovery that primate lentivirus infection leads to AIDS-like disease in macaques has allowed the development of models for key aspects of HIV-1 infection in humans. Specific combinations of lentiviruses in different hosts have led to a number of observations that have confirmed or informed HIV-1 infection of humans. Using these models, certain types of studies can be performed that would be risky or unethical to pursue in human clinical studies. A summary of the major nonhuman primate lentivirus infection models is shown in table 1, noting the nonhuman primate common name, the viruses tested, and the concepts elucidated by these models.

From our 21st century perspective, we can see that the models that result in pathogenesis represent infection in non-natural hosts, where the virus is by definition poorly adapted. One of the first models to be utilized was the infection of chimpanzees *(Pan troglodytes)* with HIV-1, using lab-adapted viruses that were the only ones available at the time: HIV-IIIB/LAI and HIV-SF2. This model was useful in recapitulating the infection process, route of infection, and antiviral immunity established. It was only after more than 10 years of study that disease was observed[4]. Given the knowledge that HIV arose from chimpanzees[24], it is less surprising that the human-adapted HIV-1 strains were not very pathogenic when reintroduced into the chimpanzee. For comparison, we need look only at the African simian lentiviruses in their native hosts. SIVsm infection of sooty mangabeys[25], much as SIVagm infection in African green monkeys[26], shows high replication without pathogenesis, evidence of a virus that is well adapted to the host. HIV-1 originally looked promising in the pigtailed macaque[27], but viral replication was not sustained and pathogenesis did not ensue.

Table 1. Pathogenesis and immunity in nonhuman primate lentivirus models

Natural host	Virus	Concepts elucidated (reference)
Sooty mangabey	SIVsm	– Significant viral replication in natural host[91,92] – Lack of pathogenesis due to adaptation or lack of immune activation[25]
African green monkey	SIVagm	– Significant viral replication[20,94] – Lack of pathogenesis due to adaptation[26]
Experimental host	**Virus**	**Concepts elucidated (reference)**
Chimpanzee	HIV-1	– Time to disease similar to humans[4,95,96] – Superinfection observed by multiple HIV-1 clones[97] – Infection by mucosal and intravenous routes[98] – Immunity: T-cell and B-cell immunity[23,99-101]
Pig-tailed macaque	HIV-1	– No sustained replication and no pathogenesis[102,103]
Baboon	HIV-2	– Relatively low-level replication with no sustained disease[5,22,104]
Macaque species	SIVmac, SIVmne, SIVsm	– Progression to disease typically accompanied by loss of CD4+ T-cells[105] – Replication dependent upon virus isolate or clone[3] – Time to disease predicted by plasma virus load[32,33] – Roles of individual genes in infection, pathogenesis in vivo[106,107] – Breakthrough SIV or SHIV after sustained control[108,109] – Mucosal transmission and fate of infecting virus[110-112] – 100-fold more virus required for mucosal vs. intravenous infection[113,114] – Tissue tropism of certain viruses and clones[115] – Coreceptor studies[116-118] – Rapid disease progression in newborns[119] – Viral diversification from clonal infection[120,121] – Escape from CTL[122,123] – Escape from NAb[123-125] – Removal of CD8 cells results in viral rebound[35]; B-cells also important[37]
Macaque species	HIV-2, SHIV (X4)	– Rapid, irreversible CD4+ T-cell decline after multiple passages in vivo[7,126,127]
Macaque species	SHIV (R5)	– Variable set-points similar to SIV[11] – No rapid CD4+ T-cell decline – Vaginal infection

The next step toward finding a host for human viruses was to test HIV-2 in baboons and macaques, which was done with varying degrees of success. Disease was seen in the baboon model with HIV-2-UC 2[5,28], and an animal-passaged HIV-2-EHO replicated to very high levels and caused reproducible loss of CD4+ T-cell in pigtailed macaques[6]. The mechanism of rapid CD4+ T-cell loss is not well understood.

Most of the progress in studying pathogenesis and immunity has arisen from the study of African SIV in the Asian macaques: rhesus *(Macaca mulatta)*, pigtailed *(M. nemestrina)*, and crab-eating macaques *(M. fascicularis)*. There are two major phylogenetic groups, the SIVmac and SIVmne viruses, and the SIVsm viruses, which are closest to HIV-2 in sequence homology[29]. In these models, disease is accompanied by loss of CD4+ T-cells and death is usually, though not always, dependent upon acquisition of an opportunistic infection. SIV infection of macaques proved that pathogenicity was dependent upon the virus, whether clonal or non-cloned viral isolate, and upon the host[30]. The role of individual genes in pathogenesis could be directly tested, leading to a better understanding of regulatory genes such as *nef*[31]. A major step forward in validating nonhuman primate models was the discovery that plasma viral load predicts time to disease[32,33], similar to the finding that plasma viral loads are the key variable for disease progression in HIV-infected patients[34]. This correlate has allowed the evaluation of therapies and vaccines that are unable to provide "sterilizing immunity", but do affect the set-point of viral load. The relative importance of CD8+ T-cells in controlling early acute infection was directly demonstrated by depletion in vivo[35,36], and subsequently the role of neutralizing antibodies was also explored by B-cell depletion[37]. Neutralizing antibodies were shown to directly reduce the in vivo infectivity of HIV-1 in macaques, using IgG from an HIV-infected

chimpanzee (HIVIG)[38]. The development of chimeric SHIV clones bearing HIV *env* genes in the SIV backbone has allowed the testing of vaccines and therapies that are directed at the Env protein. One of the pathogenic SHIV models (SHIV-89.6P) also shows the rapid CD4+ T-cell depletion seen with macaque-passaged HIV-2-287[39]. These studies, and other similar studies with therapies and vaccines described below, underscore the power of the nonhuman primate models in understanding key immune responses in vivo.

Successes with antivirals and immune-based therapies

Antiretroviral drugs have been tested to a limited degree in nonhuman primates, and many of these studies followed FDA approval of compounds for human use. Conservation of reverse transcriptase (RT)[40] meant that RT inhibitors could be tested using SIV. Monotherapy with zidovudine (azidothymidine, or AZT) was only poorly effective in controlling acute SIV infection, and this outcome was interpreted as a weakness of the primate models[41]. Viewed in hindsight, and in comparison with more effective drugs such as protease inhibitors or with cocktails in acute infection, clinical experience with AZT has been similarly disappointing. However, there are three key discoveries in therapeutics that stemmed from successes in nonhuman primate models. It was the successful testing of (R)-9-(2-phosphonylmethoxypropyl) adenine (PMPA) by Tsai, et al.[42] that led to the development of this drug for humans, in contrast to most other antiretrovirals, which were developed on the basis of in vitro testing prior to phase I trials in humans.

The second advance was immediate postexposure prophylaxis (PEP) with stavudine (D4T) in the HIV-2-287 model. Prior to this point, there was epidemiological evidence that PEP in humans using AZT could prevent infection[43]. The macaque study showed that short-course prophylaxis at very high doses, followed by cessation of therapy, was effective in controlling viral load and preventing CD4+ T-cell decline in five out of six treated animals for more than a year after withdrawal of treatment[6]. Control of viremia and prevention of CD4 declines correlated with seroconversion, soluble CD8-produced factors, and Class I-associated control. These results demonstrate that early antiviral intervention, even of a limited duration, may constitute an important strategy against lentiviral-induced disease if antiviral immunity is present. A number of additional studies have explored the limits of very early and interrupted treatment in the SIV model[44,45] – studies that have been more difficult to perform in humans[46]. In both the human and the macaque studies, early control of viremia is important, and in cases where infection is established, host immunity is necessary for viral containment in the absence of drug[47-49].

A third advance has been in defining the potency and role of neutralizing antibodies in preventing and limiting infection. Animal models have allowed the testing of polyclonal and monoclonal preparations, both as preexposure and postexposure therapies (Table 2), as summarized in a recent review[17]. Originally these experiments were performed with human polyclonal HIVIG[50] or V3-region-specific monoclonal antibodies[51] in HIV-1-infected chimpanzees or macaques, to demonstrate sterilizing immunity. With the advent of SHIV viruses that not only replicate, but also cause disease in macaques, it has been possible to study the role of human monoclonal antibodies and polyclonal preparations as PEP and therapy. These studies demonstrated that complex neutralizing antibodies can limit the infectivity of HIV in vivo[38]. PEP therapy with polyclonal SIVIG at a high dose can ameliorate SIV infection and delay disease[52]. Parameters such as timing[53,54], potency of antibody combinations[55] and routes of challenge[14,15], and dose[56] can be explored without risking human lives. These advances have led to the testing of HIVIG[57,58] and planned testing of monoclonal antibodies as therapeutics for mother-to-child transmission in humans[59].

Vaccine protection and immunity

Given some of the useful information derived from nonhuman primate models in the therapeutic area, it is at least theoretically possible that one or more of these models may inform vaccine design. Two types of readouts are used in nonhuman primate work: vaccine-elicited immune responses and protection following viral challenge. As noted in table 1, the quality and magnitude of antiviral immune responses to HIV, SHIV and SIV infection in the non-adapted host have many commonalities. In the absence of proven correlates, an indication of the congruence of data between humans and nonhuman primates is the direct comparison of products in the two systems, and the use of validated assays to compare the immune responses. For a system to be useful as a screen, this minimal criterion must be met. Considerable efforts have been applied to the development of standardized immunological assays that can be used for both human and macaque

Table 2. Examples of therapeutic approaches tested in nonhuman primate models.

Type of study	Host	Virus	Outcome or concept (reference)
D4T treatment	Pigtailed macaque	HIV-2-287	– Immediate prophylaxis controls infection[6]
Antiretrovirals	Pigtailed macaque	SIVmne	– Postexposure chemoprophylaxis with PMPA prevents infection[42,128,129]; AZT is less effective[41] – Effects on chronic infection are poor[130,131]
Antiretrovirals	Rhesus macaque	SIV	– Transient treatment during acute infection improves outcome[45]
PMPA*, tenofovir in newborns	Rhesus macaque	SIVmac 251 SHIV-SF33	– Infection in newborns is blocked by drug treatment[132-136]
CCR5 inhibitor	Rhesus macaque	SIVmac; SHIV-89.6P; SHIV-SF162P4	– CCR5 inhibitor reduces viremia in high-replication models that use CCR5 and reduces viremia in SF162P4 infection[136]
Preexposure passive IgG	Chimpanzee	HIV-1	– HIVIG and MAbs directed to V3 can protect against HIV-IIIB (lab-adapted) challenge[50,51]
Preexposure passive IgG	Rhesus macaque	SIV, SHIV-DH12	– IgG with neutralizing activity can block infection at high doses[137,138]
Postexposure passive IgG	Rhesus macaque	SIVsmE660, SHIV-KU2 and SHIV-DH12	– Very high levels of NAb are needed to slow infection and affect disease[52,53,139,140]
Preexposure passive mAb	Rhesus macaque	SHIV89.6P	– Combinations of mAbs effective in blocking oral or vaginal infection[14,114,141-143]
Postexposure passive mAb	Rhesus macaque infants	SHIV	– Cocktails of monoclonals can block infection if given within hours of exposure[54]

*9-[2-(phosphonomethoxy)propyl]adenine.

samples. In both systems, cellular immunity is determined by antigen (peptide)-specific cytokine secretion by ELISPOT and neutralizing antibodies against panels of HIV-1 patient isolates using standard cell line-based assays. Although these assays are not yet to the stage of good laboratory practice (GLP) validation, they are close to this threshold.

Some of the key advances learned from vaccine studies in nonhuman primate models are summarized in table 3. These include:

– the relative efficacy of different types of vaccines (subunits, live recombinant viral vectors, prime-boost, and live-attenuated);

– the merits of including individual and multiple components in the vaccine (e.g. Env only, multiple antigens, regulatory genes or proteins);

– the inclusion of cytokines as adjuvants; and

– the effects of different routes of challenge.

Will these lessons translate into appropriate choices for HIV vaccines in humans? A review of the qualitative

Table 3. Key advances in vaccine development from primate models.

Concept	References
– Subunit Env gp120 vaccines provide sterilizing protection only with low-replication models	61-63
– Subunit Env gp160 and gp140 vaccines do not provide sterilizing immunity against SIV	144
– Prime-boost vaccines provide sterilizing protection with low- and moderate- but not high-replication challenges	72,73,108,145
– DNA vaccines can serve as prime or boost with vaccinia virus	80
– Parenteral vaccines protect from mucosal challenge	146
– Protection with live-attenuated vaccines is dependent upon viral replication	147,149
– Cytokine (interleukin-2) as adjuvant improves DNA and recombinant vaccinia virus vaccine immunity and efficacy	148,149
– Protection from disease is improved by inclusion of both Gag and Env antigens	144,150
– Attenuated viruses that were safe in adult, juvenile macaques cause disease in newborns	151
– Adoptive transfer of SIV-naive autologous CD4+ T-cells to macaques chronically infected with SIV is sufficient to induce long-term nonprogressor status	152

Table 4. Comparison of immune responses in vaccinated humans and nonhuman primates

Vaccines in testing	Protection in nonhuman primates	Nonhuman primate immunity (reference)	Human responses in phase I trials (reference)
Env gp120 subunit (CHO cells)	Sterilizing in chimpanzees vs. HIV-1 Limited or none vs. SIV in macaques	Low level Abs and NAbs (lab-adapted)[61,64,60]	Low level Abs and NAbs (lab-adapted)[153,154] No effect on CTL; low-level proliferation[155,156]
Env gp160 subunit (baculovirus)	No protection from SIVmac251 infection	Low-level Abs and NAbs (lab-adapted)[63]	Transient increased T-helper responses in infected patients[157,158]
Recombinant vaccinia virus expressing Env gp160	No protection in chimpanzees	Strong CTL responses[159]	Responses similar to primates but limited by prior vaccinia exposure[70]
Recombinant NYVAC *gag-pol-env* with DNA	Control of SIVmac251 infection	CD4 and CD8 responses[160]	NT
Recombinant MVA, Gag-Pol plus Env; multi-epitopes and Tat, Rev, and Nef	Attenuated viremia; SHIV-89.6P in macaques; poor protection against SIVmac239	CTL at day of challenge; post infectioncellular and humoral immunity[161,162]	NT
Canarypox *gag-pol-env* plus Env gp120 subunit	Protection from SIVmac251 disease	CD4+ and CD8+ T-cell responses[80]	Canarypox only; very weak ELISPOT responses and NAbs[153,163]
Multi-epitope CTL vaccines (peptide and lipopeptide)	None	Weak responses to Gag and Nef in majority of animals[164,165]	Weak responses in 9/12 by CTL; in 5/6 by ELISPOT[166]
Replication-incompetent adenovirus expressing Gag	Attenuated infection with SHIV challenge	Strong cell-mediated responses[67]	Significant CTL responses by ELISPOT[167]
Replication-competent adenovirus expressing Gag and Env	Protection from SIVmac251 infection and disease in macaques	Antibody and cell-mediated responses correlated with better outcome[168]	NT
Enhanced DNA vaccines; DNA/PLG microparticles	NT	Strong CMI by CTL and ELISPOT to Gag, Env[169]	Trial underway in 2004
Oligomeric Env gp140 subunits and deletion subunits	Reduction in viremia with SHIV challenge	NAbs against primary and lab-adapted HIV[169] and some heterologous primary HIV (deletion mutants)[170,171]	Trial underway in 2004

and quantitative immune responses elicited by different and similar vaccines yielded a surprising degree of congruence for some assays and for many of the approaches tested to date, as summarized in table 4. The list of vaccines that have been tested in both systems is increasing, and the examples shown here are illustrative rather than comprehensive.

The earliest vaccine approaches were focused on the recombinant Env subunits gp120 and gp160. In primates, these had shown some ability to elicit neutralizing antibodies against laboratory isolates, and limited activity against primary HIV-1. Env preparations with conformational determinants preserved was more effective in generating neutralizing antibodies in baboons than denatured non-glycosylated Env, and the magnitude of responses was adjuvant-dependent, with alum being the least effective[60]. Relative to HIV-1 infection, however, the magnitude of neutralizing antibodies was at least 10-fold lower. Although there was evidence of complete protection from HIV-1 challenge in chimpanzees[61,62], these subunits did not provide sterilizing protection in macaques using the SIV-homologous gp130[63,64], but could block or limit infection by a lower-replicating SHIV[65]. The first vaccine product to be tested in humans in an FDA-approved trial was the baculovirus-produced gp160 protein. When gp120 products were tested in humans, they also elicited neutralizing antibodies restricted to laboratory isolates, at levels at least 10-fold lower than in infected humans[66]. And when tested in phase III clinical trials, gp120 failed to achieve the 30% efficacy of sterilizing protection that the trial was powered to observe[21]. Recent efforts have been directed at testing oligomeric Env proteins that can present oligomeric and conformational determinants[67].

The first FDA-approved recombinant viral vector vaccine trial tested HIVAC-1e, recombinant vaccinia virus expressing gp160 from HIV-IIIB/LAI. This system was viewed as attractive from a number of standpoints, including the expression of native Env gp160 in vivo, which allowed induction of cytotoxic T lymphocytes (CTL) as well as antibodies, and the virus infection was

self-limiting due to anti-vaccinia clearance. The relatively weak immunity raised by HIVAC-1e in chimpanzees[68,69], and ultimately in humans[70,71], led to the development of a strategy that now is termed "prime-boost." Originally envisioned as a method to boost humoral immunity using an orthogonal antigen delivery method, macaques primed with recombinant vaccinia virus expressing HIV Env gp160 were boosted with purified Env gp160 glycoprotein, which increased antibody responses. When challenged with SIVmne, these macaques fully resisted infection[72]. Unfortunately, more stringent SIV-challenge models failed to show sterilizing immunity[63,73]. Results in humans were similarly weak, showing some cellular responses and neutralizing antibodies against laboratory isolates[74-76].

Safety issues with vaccinia virus in humans, as well as evidence that macaques[77] and persons[70] with preexisting vaccinia immunity were poor responders, led to explorations of more attenuated poxvirus vectors[78,79], including modified vaccinia Ankara (MVA) and Avipox or canarypox vectors[80,81]. When used alone, these vectors were effective in eliciting CTL[82] and, in conjunction with protein boosting, were effective in limiting postchallenge pathogenesis[83,84]. In humans, immunity was detectable but weak[85]. Other strategies aimed at presenting specific CTL epitopes alone, or in combination as multi-epitope vaccines, have elicited weak responses, both in nonhuman primates and in humans.

Alternative recombinant adenovirus vectors, either one-round or replication competent, are also showing significant promise in macaques and in humans in generating both humoral and fairly strong cellular immunity[68,87]. Experience to date with these two types of adenovirus is shown as an example in table 4[88]. Where comparative data are available, they show similar levels of immunity elicited. DNA vaccines have the potential of generating broad responses against multiple antigens with relative ease. The first generation of these have been tested fairly extensively in macaque SHIV-challenge models, reviewed in[69], and these experiments show the value of combining two or more vaccine types[90]. The relatively weak immunity elicited by DNA vaccines in macaques and in humans suggests that better adjuvants and delivery systems are needed, and some of these are showing promise in nonhuman primates. DNA complexed inside, or on the surface of PLG microspheres, reduces the amount of DNA needed per immunization. This strategy has entered human phase I testing in conjunction with a modified oligomeric Env gp140 subunit that has shown promise in nonhuman primates in eliciting primary HIV-1 neutralizing antibodies. If these newer recombinant viral vectors, DNA delivery systems, and oligomeric protein strategies elicit improved immunity in humans, this will be additional supportive data for the predictive value of nonhuman primate testing in vaccine development.

Remaining Issues

We are faced with several critical challenges in the control and prevention of HIV infection and disease. In infected humans, the virus causes extreme suffering and 100% mortality. Effective nontoxic drugs are still desperately needed for those who become infected and to more effectively prevent mother-to-child transmission. If vaccines are to protect against any of the multiple genetic variants that continue to diversify and recombine worldwide, persistent, broad cellular and humoral immunity are both needed. None of the vaccine candidates currently in testing comes close to eliciting the level of responses seen in infected individuals such as long-term nonprogressors.

The data summarized here suggest that, where beneficial effects of drugs, immune-based antiviral therapies, and vaccines, are seen in the nonhuman primate models, these may be indicative of potential success in humans. Clearly there are limitations to the use of nonhuman primate models, particularly in enzymatic drug targets that differ between SIV and HIV-1, such as with protease. Are models that cause rapid, irreversible CD4+ T-cell decline representative of HIV, with its steady slow decline over years? It is also not yet clear whether SHIVs are representative of HIV-1 in their pathogenic course. No single animal model is likely to serve as a perfect model for HIV infection of humans. Each of the models has advantages, but none can replace the knowledge gained from human clinical work. As a field, most investigators agree that we are still years away from having nontoxic drugs that will effectively control infection, or a vaccine candidate that will provide even modest protection from disease. Currently, testing in nonhuman primates is not considered to be on the critical path for drug or vaccine testing. However, there are compelling arguments for a parallel pathway of discovery and testing in nonhuman primates. Testing of orphan drugs such as PMPA proceeded in humans only after successful testing in nonhuman primates – an example of the use of positive data in the model systems to ignite enthusiasm for clinical testing. As antiretrovirals become more widely available and become the standard of care, it may be difficult to design low-cost trials to test new products;

primate testing can complement or augment these findings by the ethical testing of new components, such as monoclonals, for efficacy in the absence of the current standard-of-care.

Should nonhuman primates be on the critical path for vaccine testing? The published experimental data for vaccines show that the quality and magnitude of immune responses elicited in macaques is in many cases similar to that seen in humans, and thus may be at least relatively predictive of responses in humans. It would be risky to extrapolate vaccine success based solely on results of challenge studies in nonhuman primates. However, given the surprising congruence of the immunogenicity data, it can be argued that the vaccine successes we have seen in the nonhuman primate models may portend the ultimate success for human vaccines that can blunt infection, if not prevent it. Vaccines with strong safety profiles that are successful in protection from disease or infection in more than one nonhuman virus-host model should be considered first for testing in humans, as they may ultimately lead to successful HIV vaccines. Advances in science are derived both from individual breakthroughs as well as the combined wisdom of multiple concordant studies that define limits and show reproducibility. It will take new ideas as well as the continued collaborative efforts of the entire field to control this difficult and challenging pathogen. The nonhuman primate models for AIDS remain a critical tool in this endeavor.

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Assessing animal models of AIDS

C22

Animal models of AIDS continue to surprise researchers with results that can be difficult to explain. However, most researchers still believe that animal models are essential to progress in understanding the disease. But which animal model is best? (pages 321–329)

JAMES STOTT & NEIL ALMOND

A report published in this issue of *Nature Medicine* by Abimiku *et al.* asserts that vaccination with components of HIV-1 protects macaques against challenge with HIV-2 (ref. 1). This unexpected finding challenges many of our prejudices about how to vaccinate and protect against immunodeficiency viruses. When faced with surprising and possibly unwelcome news, there has been a tendency in AIDS research to 'shoot the messenger.' As a result, animal models have been condemned as unreliable, inappropriate and even misleading. The reality is that all animal models are potentially capable of yielding relevant information, provided that experiments are appropriately designed and well controlled. As in all scientific endeavour, individual observations need independent confirmation before they are accepted as established fact. If the results of *in vivo* experiments in different animal models appear to conflict with each other, with results of *in vitro* work or with accepted preconceptions, then this may reflect our ignorance of the biology of immunodeficiency viruses.

The observations reported by Abimiku *et al.* are intriguing, but essentially very preliminary. A group of three naive control animals is compared with eight vaccinated macaques, each of which received a unique immunization with one of several different cocktails of antigens. Hence, as the authors acknowledge, even when the disparate vaccinated individuals are treated as a single group, these results do not reach statistical significance and could be a consequence of chance. The report thus illustrates a common difficulty encountered with animal models for AIDS: the limited numbers of individuals allocated to test and control groups.

Four different types of model have been used in AIDS research: chimpanzees infected with HIV-1, macaque monkeys infected with either SIV or HIV-2, cats infected with feline immunodeficiency virus and SCID mice immunologically reconstituted with human cells and infected with HIV.

The use of non-human primates such as chimpanzees or macaques is constrained by generally accepted practical, economic and even ethical limitations. As a result, undue scientific credibility has often been given to the protection against infection of very small numbers of non-human primates, numbers that defy the application of conventional statistical analysis. This has been particularly true of results obtained in chimpanzees. It is clearly important that results must be subjected to appropriate statistical analysis whenever possible and extreme caution exercised when this is not the case. However, more credibility is due where the studies form part of a logical sequence of investigation with a consistent theme and outcome. One solution to the problem has been to use a standardized stock of challenge virus so that the accumulation of historical controls gives greater confidence in the validity of protected vaccinated individuals[2]. Most primate centres in the United States and Europe have been commendably generous in sharing well-characterized stocks of challenge viruses. Those working with macaques have also sought to pool resources and to design larger collaborative experiments using standardized vaccines and challenge viruses[3]. Nevertheless, the numbers of macaques that have been used in AIDS vaccine development is relatively small when compared with the numbers that were used in the 1950s and 1960s in the development of poliovirus vaccines.

When faced with surprising and possibly unwelcome news, there has been a tendency in AIDS research to 'shoot the messenger.' As a result, animal models have been condemned . . . .

Animal models for AIDS are a crucially important resource with which to understand the biology of immunodeficiency viruses *in vivo*, and thus to gain essential insights into the mechanisms of pathogenesis and its prevention by vaccination and chemotherapy. In the twelve years since HIV was isolated, an impressive volume of knowledge has accumulated on the genetics and molecular biology of lentiviruses, but we remain largely ignorant of the biology of pathogenesis and strategies for prevention of this disease. *In vitro* experiments in cell culture may provide insights and clues, but there is no substitute for experiments *in vivo*, particularly with a virus that is so intimately involved with the complex cellular interactions of the intact immune system. Much has been learned about pathogenesis from the intensive study of HIV-infected patients. However, crucial early events in infection can only be investigated in animal models where timing, dose, route and nature of the challenge virus can be precisely controlled, and where the distribution of virus in a wide range of tissues can be assessed. Many antiviral therapies have been evaluated in clinical trials in infected volunteers. Nevertheless, animal models make a unique contribution to the evaluation of drugs administered immediately after exposure. Non-human primates also contribute to the evaluation of therapies involving immune modulators where the outcome is too uncertain to risk immediate use in humans. Vaccine strategies can only be assessed *in vivo*. Phase I and II human clinical trials have successfully tested the safety and antigenicity of candidate vaccines. However, phase III efficacy trials will be costly. Thus, efficacy data from animal models are crucial for identifying the *in vitro* immune correlates of protection and deciding which vaccines should proceed to phase III trials. Many candidate vaccines look promising until they are exposed to the harsh reality of a live virus challenge *in vivo*.

The use of chimpanzees remains controversial. They are a rare and protected species. Therefore, infected animals must be kept in containment for the duration of their natural life, and now require a pension fund in excess of

US$100,000 per individual. Although these animals are readily infected by certain strains of HIV-1, replication is more limited than in humans and, so far, no animals have developed significant immunodeficiency disease. Hence, this model is inappropriate for the study of pathogenesis or the prevention of disease by vaccines or antiviral drugs. However, if the objective is to test the efficacy of a candidate HIV-1 vaccine against infection, then the chimpanzee is an appropriate animal model, and has been successfully used for this purpose. Vaccination with various HIV-1 envelope preparations and peptides has protected a number of chimpanzees against challenge with the homologous LAI strain[6,7] or heterologous SF2 strain of HIV-1. Passive transfer of antibody to the V3 region of the envelope has also protected[8]. Caveats that must be observed in interpreting these results are (1) none of the individual experiments so far reported have reached statistical significance, (2) the challenge viruses are laboratory-adapted strains and (3) because the replication of HIV in chimpanzees is more restricted than in humans they may be more easily protected, and thus overestimate the efficacy of a vaccine designed for use in humans.

Infection of reconstituted SCID mice with HIV carries the same limitations as chimpanzees. Virus replication is limited and no immunodeficiency disease develops. In addition, the SCID mouse is a highly artificial animal. Despite these reservations the system has provided insights into the immunity of human volunteers vaccinated with HIV-1 vaccine whose serum or cells have subsequently been transferred to SCID mice, protecting a proportion against HIV-1 infection. It remains to be seen how far this elaborate organ culture system will predict the true efficacy of AIDS vaccines in humans.

Feline immunodeficiency virus infection of cats is a naturally occurring disease with its own economic importance. Hence, studies of pathogenesis and development of vaccines against this infection have their own intrinsic and commercial value, in addition to their value as a model for AIDS. Sufficient numbers of cats may be used to provide statistically significant results. However, the virus is significantly different from HIV-1 genetically, and it enters cells via a different receptor. The immune system of the cat is also substantially different from that of primates. Despite these limitations, insights obtained about FIV infection of cats and its prevention by vaccination are likely to be relevant to HIV infection in humans, and thus have added value to the need for a feline vaccine. Furthermore, if the same principles of pathogenesis and protection emerge from a variety of diverse animal models, confidence in their validity and relevance to human AIDS will be increased.

The reality is that all animal models are potentially capable of yielding relevant information, provided that experiments are appropriately designed and well controlled.

Macaque monkeys remain the most widely used animal model for AIDS. They can be infected by a variety of strains of SIV or HIV-2 which range from the highly virulent PBj14 strain of SIVsm to the attenuated infectious molecular clones of SIVmac carrying deletions in *nef* or other non-essential genes. Hence, precise studies of pathogenesis may be undertaken in which early events can be analysed in detail using a variety of viruses in which individual genes may be modified to define their role in virulence. Furthermore, vaccine strategies can be assessed for their ability to prevent virus infection, to reduce virus load, or to prevent or delay the development of disease. In all of these studies, adequate numbers of animals may be used to provide statistically significant data. SIV, like HIV, uses the CD4 receptor and is genetically closer — though not identical — to HIV-1 than any other vertebrate lentivirus. A further advantage of this model is that many of the human immunological markers and immune modulators are equally effective in the simian immune system.

The majority of macaques protected against infection were immunized with inactivated SIV[9], but it is now clear that the protection was induced predominately by cellular antigens — not viral antigens — in the vaccine[9]. Vaccination of macaques with SIV envelope protein reduces virus load early after infection[9,10] and may result in clearance of the infection and subsequent delay or prevention of disease. Complete protection against infection is only observed when the challenge virus has restricted replication *in vivo* as with SIVmne[11].

By far the most potent protection so far observed in macaques is induced by attenuated virus[12]. Animals are protected against high doses of challenge virus given intravenously or intrarectally, and against challenge with intact virus-infected cells. The macaque is an ideal model in which to study attenuation of these viruses and the mechanisms by which they protect. A major limitation of the macaque model is that SIV and HIV-2 are not HIV-1, making it is a surrogate model. However, even this problem may be partially overcome by the use of chimeric viruses in which individual genes of SIV are replaced by those of HIV-1[13].

There are precedents for unwelcome results from the SIV macaque model. These were initially thought to be misleading, but they have helped to advance knowledge and to rectify generally held misconceptions. Some examples serve to illustrate this truth. First, early studies of the neutralization of HIV-1 laid great emphasis on the predominant importance of the V3 loop of the envelope protein. Since antibodies to the V3 region of SIV did not have neutralizing activity, the SIV macaque model was deemed to be irrelevant in HIV vaccine development. Subsequent studies on clinical isolates of HIV-1 suggest that the predominance of V3 may be an artefact of *in vitro* passage, indicating that the mechanism of neutralization is closer to that demonstrated for SIV than to the highly laboratory adapted IIIB strain of HIV-1 used in earlier studies. Second, the discovery in the SIV macaque model that T-cell antigens were capable of mediating protection against infection was initially condemned as a demonstration of the unreliability and irrelevance of this model in vaccine development. Subsequent studies have confirmed the observations and advanced our understanding of the intimate relationship between immunodeficiency viruses and their host cells. This discovery has also provided new opportunities for alternative approaches to AIDS vaccine development. Third, a series of *in vitro* studies on the role of the *nef* gene yielded conflicting results. The definitive *in vivo* studies by Desrosiers' group in the SIV macaque model established that *nef* is important in pathogenesis — a result subsequently confirmed by others[12,14,15].

All animal models have limitations but each will reveal essential insights that can only be obtained *in vivo*. The currently perplexing observations in this issue of *Nature Medicine* must have a logical explanation. Ultimately, we shall only know which is the best model when we understand the pathogenesis of AIDS, and when we have vaccines and therapies that prevent it. In the meantime, our own bias, on the evidence so far, is that infection of macaques with SIV or HIV-2 will prove to be the most rewarding and reliable model for pathogenesis and prevention of AIDS.

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Anatomical research lives!

A previously undescribed tissue connection may provide an explanation for some forms of tension headache. It also proves that common beliefs about anatomical research reaching its limits are unfounded.

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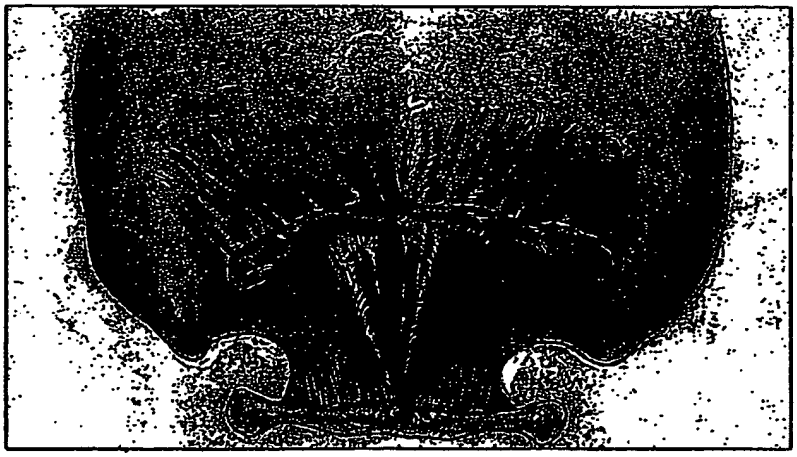
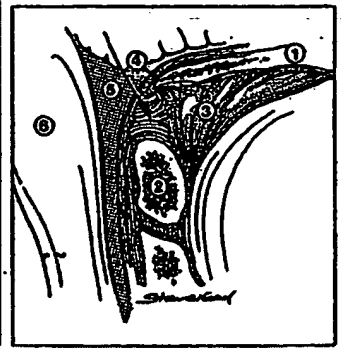
The announcement of a new anatomical finding is astonishing during these times of re-evaluation and closing of anatomy departments in medical and dental schools. This is particularly true if such a finding helps explain the relationship between headache and muscle tension, a highly controversial area. Yet this has happened. At a recent meeting of the Joint Section on Disorders of the Spine and Peripheral Nerves of the American Association of Neurological Surgeons, Gary D. Hack of the University of Maryland at Baltimore Dental School and Walker L. Robinson of the University of Maryland School of Medicine (Baltimore) presented their findings on an observed connective tissue bridge between the spinal dura and the rectus capitis posterior minor (RCPM) Muscle (see figures). This observation, submitted for publication, represents over three years of collaborative investigation by Hack and Robinson and their anatomical mentor Richard T. Koritzer, a unique collaboration between two general dentists and a neurosurgeon.

Their finding was serendipitous. It occurred during inspection of the muscles of mastication in sagittally hemisected head and neck cadaveric specimens, which is not a standard dissection approach. The research team observed the RCPM muscle-dural connection and noted that simulated contraction of the muscle tensed the dura in this area. Subsequently they have found this connection in 25 consecutive dissections, including two black males, one black female, fourteen white males and eight white females. A suitable animal model is being actively sought. This connective tissue bridge has been verified by James L. Hiatt, an anatomist at the University of Maryland at Baltimore,





Illustrations of the region in the head where a previously undiscovered muscle-dural connection is found. (above) A drawing of the deep occipital region (posterior view). The rectus capitis posterior minor (RCPM) muscle is seen extending from the first cervical vertebrae (C1) to the occipital bone. Superficial musculature has been removed.
(left) A drawing of a hemisected head and neck specimen. 1, occipital bone (posterior border of foramen magnum); 2, atlas (posterior arch); 3, RCPM muscle; 4, dura mater; 5, cerebrospinal fluid in cisterna magna; 6, spinal cord. Arrow, connective tissue bridge linking the RCPM muscle to the dura mater.

(COURTESY OF TOM STEVENSON, UNIVERSITY OF MARYLAND AT BALTIMORE)